Sustainable Management of Tannery Hair Waste through Composting

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ABSTRACT

Bovine hair, resulting from the unhairing of hides and skins, forms a major component of tannery solid wastes that is often disposed to landfill. However, following increasing environmental legislation restricting the disposal of commercial and industrial organic wastes to landfill, alternative treatment of tannery solid hair waste is necessary. The overall aim of this research was to develop conditions favourable for the decomposition of bovine hair waste in a composting environment. In this study, chemical pretreatments of bovine hair were carried out to enhance susceptibility to enzymatic digestion. Pretreatment using a combination of potassium thioglycolate and sodium hydroxide enhanced enzymatic digestibility of hair. Comparative solubilisation of black, brown and white hair samples with sodium sulfide showed that, the percentage soluble nitrogen, from white hair was 73% (w/w) as compared to 28% (w/w) and 24% (w/w) of black and brown hair, respectively; indicating that white hair was more vulnerable to solubilisation.

Although chemical pretreatment was effective, it was considered non-compatible with the composting process. Consequently, enzymatic and microbial degradation were considered suitable alternatives. The results of the preliminary biochemical studies indicated that, the breakdown of the hair structure was enhanced when treated with a combined protease solution containing proteinase K and trypsin enzymes as compared with individual enzymes. Enzymatic and microbial degradation of hair was observed to be preferential, starting with the least resistant components, in this sequence: (1) cell membrane, (2) endocuticle, (3) medulla, and (4) cytoplasmic material and fibrillar components of the cortex. The cuticle was most resistant to degradation. Synergistic action of microbial-expressed enzymes was observed to be the fundamental mechanism for the enhanced hair degradation.

Composting of bovine hair, mixed with dry leaves, soil, and wood chips/sawdust was performed in a rotary drum under controlled conditions. Optimum conditions, in order to sustain thermophilic microbial flora, were found to be (1) a moisture content of 55±2% RH, (2) pH 7 – 8, (3) temperature of 49±2 °C, and (4) a carbon to nitrogen ratio of 35:1. Structural changes in hair, assessed during composting using scanning electron microscopy (SEM), indicated that degradation occurred under these conditions. Microscopy results further indicated that the addition of an inoculant, containing a microbial crude enzyme, expressed by the bacteria of *Bacillus* species, improved the biodegradation of hair during the controlled composting process.

The compost stability evaluated by monitoring the humification parameters and carbon to nitrogen ratio, indicated that the final product reached the following values: degree of humification (73 – 78%), humification ratio (26 – 32%), humification index (0.29 – 0.35), and a carbon to nitrogen ratio (25 – 29). The high level of humification of the end-product indicated reasonable stability, suggesting the product from the composted bovine hair may be suitable for agronomical purposes.

The use of composting as alternative technology for the treatment of tannery solid bovine hair waste, as a value added product, has been demonstrated.

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LIST OF ABBREVIATIONS

ABPR	Animal By-Products Regulation
AEBSF	4-(2-aminoethyl)-benzenesulfonyl fluoride
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
BAT	Best Available Techniques
BOD	Biological Oxygen Demand
BSA	Bovine serum albumin
BSLT	British School of Leather Technology
BREFs	Reference Documents (based on EU's BAT)
CMC	Cell membrane complex
C:N	Carbon to nitrogen ratio
CO_2	Carbon dioxide
COD	Chemical Oxygen Demand
COM	Communication
C_{org}	Organic carbon
COTANCE	Confederation of National Association of Tanners of the European Community
DH	Degree of Humification
DHI	5-6-dihydroxyindole-2-carboxylic acid
DSC	Differential Scanning Calorimetry
DTT	Dithiothreitol
EC	European Community
E.D.T.A	Ethylenediaminetetracetic acid
EEC	European Economic Community

EMCC	European monitoring centre for change
EU	European Union
FA	Fulvic acid
FAO	Food and Agricultural Organisation
GnHCl	Guanidine Hydrochloride
HA	Humic acid
H_2O	Water
HCl	Hydrochloric acid
HI	Humification index
HR	Humification ratio
hrs	Hours
ICT	International Council of Tanners
IF	Intermediate filament
IPPC	Integrated Pollution Prevention and Control
IUE	International Union of Environment
KTG	Potassium thioglycolate
MBT	Mechanical biological treatment
min^{-1}	Per minute
NaOH	Sodium hydroxide
NaOHKTG	Sodium hydroxide-potassium thioglycolate
Na_2S	Sodium sulfide
NH	Non-humic matter
N_2O	Nitrous oxide
NO_X	Nitrogen oxide
O_2	Oxygen
OECD	Organisation for Economic Co-operation and Development

OM	Organic matter
PAGE	Polyacrylamide gel electrophoresis
r.p.m	Revolutions per minute
S–S	Disulfide bonds
Scams	*S-carbamoylmethyl* keratins
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SO_2	Sulfur dioxide
SEM	Scanning electron microscopy
TCA	Trichloroacetic acid
TEC	Total extractable carbon
Temp.	Temperature
TKN	Total Kjeldahl Nitrogen
Tris	Tris(hydroxymethyl)-aminomethane
UK	United Kingdom
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
WHO	World Health Organisation
w/v	Weight per volume

SI UNITS

cm^3	cubic centimeter (1 cm^3 = 1 ml = 1 x 10^{-3} L)
g	gram
kg	Kilogram (1 kg = 1 x 10^{-3} g)
L	Litre
mg	milligram (1 mg = 1 x 10^{-3} g)

ml	millilitre (1 ml = 1 x 10^{-3} L)
tonne	tonne (1 tonne = 1 x 10^{3} kg)
µg	microgram (1 µg = 1 x 10^{-3} mg)
µl	microlitre (1 µl = 1 x 10^{-3} ml)

CHAPTER 1

INTRODUCTION

CHAPTER 1

1. INTRODUCTION

The leather industry generates substantial amount of solid hair waste, which arises during the routine industrial unhairing (hair removal) by hair recovery processing methods. Quite often, the recovered hair is disposed in landfills (Frendrup, 2000). However, environmental concerns, particularly in the developed countries, have led to increasing demand to reduce the disposal of organic wastes to landfill (Directive 99/31/EC; USEPA, 2002). It is believed that the potential use of tannery hair waste is as a fertiliser due to its high nitrogen content (Cantera and Buljan, 1997; Money, 1991; White *et al.*, 1991). Composting has been proposed as the most suitable treatment for the recovered tannery hair waste (Money, 1991). However, keratin, which is the main constituent protein of hair, is highly resistant to chemical and enzymatic degradation (Fraser *et al.*, 1972), making the biodegradation of hair to be slow and inefficient under natural conditions (Gupta and Ramnani, 2006). Composting has been widely accepted as an environmental friendly alternative way of recycling organic solid waste (Insam and De Bertoldi, 2007; Stentiford, 1987). Currently, there is little information concerning composting of hair waste. Thus, this thesis aims to address the issue.

This thesis is divided into six Chapters. Chapter 1 gives the general background information about the leather industry: waste generated and environmental impacts, waste management and the relevant legislations, a review of the biochemical properties of hair, and the overall aim and objectives of the research. Chapter 2 looks at the appropriate pretreatment for hair and the mechanism of hair degradation. Chapter 3 explores a biological (enzymatic) approach in the degradation of hair as a possible option for improving the breakdown of hair. The outcomes may lead to the development of a biological mixture with the capability of efficiently breaking down hair. Chapter 4 of this thesis is concerned with the identification of micro-organisms with the potential to degrade keratin, a general discussion of the mechanism of degradation, and the study of optimum conditions at which the micro-organisms can be utilised in a composting environment to enhance hair degradation. Chapter 5 outlines the composting experiments for bovine hair, the key parameters, and the use of an inoculum to improve the biodegradation of hair. The conclusion and future work are discussed in Chapter 6. The conclusion draws from the discussions from the previous Chapters, the key findings and their interpretation. The discussion

on future work gives few perspectives on potential opportunities and improvements in this field of research.

1.1 The global leather industry

1.1.1 General overview

Raw hides and skins, which are traditionally processed into leather, constitute a substantial part of the by-products from the meat industry. The tanning industry, therefore, plays an important environmental role by salvaging and adding value to these by-products of the meat industry which would, otherwise, pose a major waste disposal problem (COTANCE, 2002). The latest estimates by the Food and Agricultural Organisation (FAO) on the global production of raw hides and skins are represented in Table 1.1 (FAO, 2008).

Table 1.1: World production of hides and skins (FAO, 2008).

	Bovine hides		Sheep skins	Goat skins
	Million pieces	Million tonnes	Million pieces	Million pieces
Developing countries	232.8	3.8	383.9	368.8
Developed countries	111.2	2.5	187.5	19.1
World total	**344**	**6.3**	**571.4**	**387.9**

By processing the raw hides and skins, the global leather industry produces approximately 18 billion square feet of leather annually, estimated to value approximately $ 40 billion. The finished leather finds many applications in downstream sectors of the consumer product industry such as in the production of garments, bags, upholstery, heavy luggage and gloves. The different applications require different types of leather. Footwear remains the major end use product of leather with an estimated share of 65% of the world production of leather going into the industry. The global footwear production is around 11 billion pairs, worth about $ 150 billion. The value of leather produced for the automotive industry is estimated at $ 1.3 million (Secretariat, 2004). According to the estimates provided by the International Council of Tanners (ICT, 2009) the leather industry worldwide provides employment to nearly 500,000 people, with many more being employed in the downstream manufacturing sectors.

1.1.2 Structure and Economics of the European Tanning Sector

The European leather industry consists of about 3000 tanneries, which are mainly family owned small and medium-sized enterprises (SMEs). The industry is an important player in the international leather trade, being one of the largest suppliers to the international market, with a share of 25% of the world's leather production. Italy is the major producer in Europe, representing 55% of the total EU production, followed by Spain, which represents 16% of the total production. Italy is also the second largest global exporter of leather and leather products after China (Acharya, 2009). The tanning sector contributes significantly to the local European economy by offering employment to about 55,000 people, and a trade turnover of nearly € 8 billion (COTANCE, 2007). The production of bovine hides and skins in Europe are given in Table 1.2.

Table 1.2: Bovine hides and skins production in the European Union for the period 2005-2007 (FAO, 2008).

Year	Bovine hides		Sheep skins	Goat skins
	Million pieces	Thousand tonnes	Million pieces	Million pieces
2007	32	764.2	84.9	11.3
2006	31.1	742.8	80.4	10.7
2005	32	761.7	79.6	11.4
Total	**95.1**	**2268.7**	**244.9**	**33.4**

Hides and skins are processed into leather for use in a wide range of products across several sectors within the EU consumer markets, from automotives, furniture and fashion to footwear. Footwear is the largest consumer of the EU leather with a share of 50% of the total tanning production, followed by the clothing industry (20%), furniture and upholstery (17%), and leather goods sector (13%) according to a report by the European Monitoring Centre for Change (EMCC, 2004).

Although the leather industry performs an environmentally important activity by transforming hides and skins into leather it is potentially pollution-intensive (COTANCE, 2002). Consequently, the industry is often criticised and associated with the pollution of the environment as a result of the chemicals used and the by-products of the manufacturing processes, this is, in spite of its added economic significance and effort to supply and satisfy the consumer demands. The critics

have, therefore, put the industry under pressure to meet environmental challenges associated with leather manufacturing (El Baba, *et al.*, 1999; Chakrabarti and Ramasami, 2006). As a key player in the global leather industry, the environmental challenges facing the tanning sector has become a major concern for the European Union (IPPC, 2003).

1.1.3 Overview of the tannery operations

Tannery operations may be divided into three main stages: (1) beamhouse operations, (2) tanning, and (3) finishing. The technology of leather making comprises a series of initial major operations aimed at isolating collagen, the main structural leather making component, from the non-collagenous materials of the skin. The non-leather making materials include soluble proteins, fleshing, hair, and epidermis (Bienkiewicz, 1983; Heidemann, 1993). Approximately 20 - 25% of the raw weight of bovine hide is converted to leather. The remaining 75 - 80% makes up the wastes or waste by-products (COTANCE, 2002).

The initial operations to prepare the raw material for tanning are carried out in the beamhouse. The key processes at this stage are soaking, liming and unhairing, deliming and bating. Quite often the processes involve the application of a variable amount chemicals and a substantial volume of water depending on the nature of the raw material (Sharphouse, 1989). It is estimated that approximately 80% of the tannery waste originates from the beamhouse processes (Puntener, 1995).

1.1.4 Tannery Beamhouse processes

1.1.4.1 Soaking

The principal function of soaking is to rehydrate the skin to restore it close to its original natural state, and to remove soluble proteins and dirt, *i.e.* manure, urine and blood (Covington, 2009; Heidemann, 1993; Bienkiewicz, 1983). Usually mechanical action is applied to aid the penetration of water and to accelerate the process. It is, therefore, not unusual to find fleshing residues and loose hair debris in waste float at the end of soaking.

1.1.4.2 Liming and unhairing

Liming and hair removal are the next major steps in the leather manufacturing process. The main purpose of liming is the modification of the collagen structure and the removal of non-structural proteins. Because of the chemical differences between collagen and hair they can easily be separated without affecting each other (Covington, 2009; Heidemann, 1993; Bienkiewicz, 1983). The technology of hair removal can be divided into two main groups:

(i) Hair recovery (hair-save) methods: these are based on enzymatic destruction or modification of the epidermal tissue surrounding the hair bulb so that the hair becomes loose and mechanically dislodged, or a pretreatment with calcium hydroxide to cause immunisation followed by mild sodium sulfide treatment. Under these conditions the integrity of the hair shaft remains intact (Addy *et al.*, 2001; Christner, 1988; Covington, 2009).

(ii) Hair destruction methods: methods in which hair itself is targeted and its structure destroyed causing the hair to breakdown and dissolve. In this drastic method the most commonly used chemical agents are calcium hydroxide and high concentrations of sodium sulfide (Covington, 2009; Heidemann, 1993; Bienkiewicz, 1983, Morris, 1956).

1.1.4.3 Deliming and bating

The primary function of deliming is to reduce the alkalinity and eliminate swelling in the limed materials. Bating is an enzymatic treatment, normally carried out simultaneously with deliming; it is aimed at digesting some of the collagenous and non-collagenous residues in the skin. Pigments, hair debris and remnants around the follicle in the papillary layer are removed leaving a clean grain structure (Covington, 2009; Heidemann, 1993). The hair residues, however, remain undigested and form part of the suspended solid materials.

1.1.5 Main Characteristics of tannery wastes

The principal constituent of a particular tannery waste stream depends on the type of raw material, processing stage, and the method of processing. Generally, the composition of waste generated during the leather manufacturing process typically comprises of liquid, solid waste, and gases.

1.1.5.1 Liquid waste

The entire process of tanning is often accompanied by the consumption of large volumes of water, normally in the range of 50% - 200% of the weight of the material (Covington, 2009; Sharphouse, 1989). As an example, processing 1 tonne of raw hides using the conventional tanning techniques generates about 30 m^3 of effluent containing up to 400 kg dissolved and suspended solids (Puntener, 1995). Most of the dissolved and suspended solids found in the liquid are protein residues, and extraneous matter, hair, blood and dung, removed during the beamhouse operations (Christner, 1988, Puntener, 1995; Buljan and Ludvik, 2000). Other constituents include ammoniacal nitrogen, salts, sulfide, chromium and dyes (Martin and Parkin, 1986, Puntener, 1995). Figure 1.1 shows the percentage distribution of liquid waste generated from major processing steps.

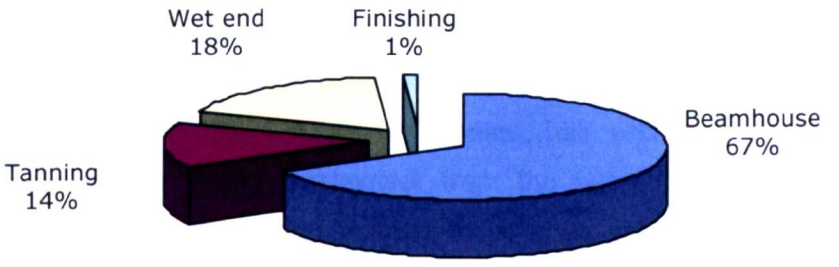

Figure 1.1: Effluent generated at each processing stage from 1 tonne of raw hides (Puntener, 1995).

1.1.5.2 Solid waste

The main constituents of solid wastes are fleshing, fats, solid hair debris, trimmings, shavings, and leather cuttings. An additional source of solid waste is the sludge from the effluent treatment plant (Buljan and Ludvik, 2000). The solid waste generated from processing 1 tonne of raw hides using the conventional processing techniques is approximately 700 kg, with the largest quantity being generated from beamhouse operations (Figure 1.2) (Puntener, 1995).

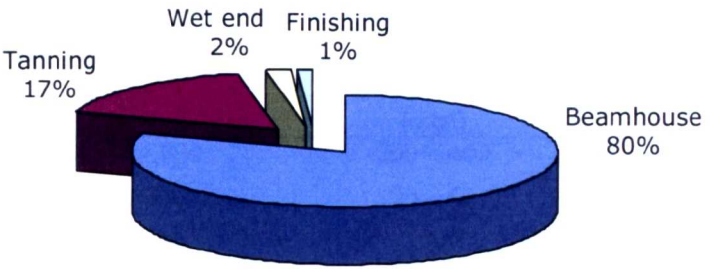

Figure 1.2: Solid waste generated at each processing stage from 1 tonne of raw hides (Puntener, 1995).

From Figures 1.1 and 1.2 it is clear that the largest source of polluting effluent in the entire tanning process, originates from the beamhouse processes.

1.1.6 Environmental impact of tannery wastes

The potential environmental impact of tanning processes concerns land, air, surface, and groundwater contamination, which by extension includes health and safety aspects (IPPC, 2003; Buljan and Ludvik, 2000). Solubilised proteins, hair and their degradation products constitute the main discharges to wastewater from the beamhouse operations leading to approximately 80% of the organic load, measured as chemical oxygen demand (COD) and biochemical oxygen demand (BOD). It is also the main source of suspended solids (IPPC, 2003; Puntener, 1995; World Bank Group, 2007). As an example, Table 1.3 represents typical effluent levels for mixed untreated tannery wastewater versus wastewater treatment efficiency, for the conventional process liquors for the production of one tonne of raw hides to finished leather. The values are compared against EU permissible limits in drinking water according to the Council Directive of 15th July (1980) relating to quality of water intended for human consumption (Directive 80/778/EEC), and urban wastewater treatment (Directive 91/271/EEC). Direct discharges of raw tannery wastewater contain an effluent with a COD and BOD concentration 100 and 200 times above the accepted levels in drinking water, respectively. Similarly, as shown in Table 1.3, typical effluent discharges from conditions considered of good practice, COD and BOD parameters are 3 and 2 times above the acceptable limits, respectively. Under these conditions, the concentration of nitrogen is 10 times the permissible level in potable water. The overall potential risks under these conditions is the contamination of surface and groundwater leading to toxic water and eutrophic conditions, posing health risks to animals and humans (WHO, 2002, COTANCE, 2002; Buljan and Ludvik, 2000).

Table 1.3: Effluent levels from tanning processes.

Parameter	Composite untreated tannery wastewater[a] (mg/l)	Typical discharge of tannery waste water treatment[b, c] (mg/l)	Acceptable guide limits[d, e] (mg/l)
COD	2,400 – 14,000	200 – 400	125
BOD	900 – 6,000	20 – 60	25
Total nitrogen	200 – 1,000	150	10 –15 µg/l
Sulfide	200 – 800	2 – 10	Undetectable
Suspended solids	~2,800 – 13,500	20 – 50	<35

Sources: (a) World Bank Group (2007), (b) IUE (2008), (c) IPPC (2003), (d) Directive 80/778/EEC, and (e) Directive 91/271/EEC

Air pollution as a result of gaseous emissions is associated with; ammonium salts used during deliming/bating process, sodium sulfide used in the unhairing process (which can be transformed to the highly toxic hydrogen sulfide gas), and post-tanning operations. Exposure to hydrogen sulfide gas is known to cause headaches, nausea and eye irritation, with some fatalities having been recorded (COTANCE, 2002; WHO, 2003). An additional source of air pollutants originates from processing activities using biocides, surfactants, and dry-finishing processes (COTANCE, 2002). Potential emissions of nitric oxide when organically-bound nitrogen is oxidised during incineration have become an increasing concern. The nitrogen in tannery solid waste is contributed by hair and the large amount of proteinaceous materials (Sarofim, 1977). Due to the considerable environmental threat from the aforementioned outputs, many countries, particularly those in the European Union (27 countries), has adopted a wide range of legislation on waste management, backed up with guidelines on specific manufacturing processes based on best available techniques (Directive 2006/12/EC).

1.1.7 Current disposal practices of tannery waste

The most common way of disposal of much of the tannery waste is by landfill (Hughes, 1988). Annually, the European leather industry disposes of in excess of 1 million tonnes of proteinaceous waste to landfill, which includes fleshing, leather shavings and about 1.2 million tonnes of sludge from effluent treatment plants which contain chromium (Hughes, 1988; COTANCE, 2002; IPPC, 2003). The legislative demand to reduce the volume of organic waste to landfill is compelling the industry to consider other waste management options.

1.2 Waste management

1.2.1 Introduction

Historically the need for waste management regulation was initiated following concerns to public health and environment, triggered by increased volume of industrial and domestic waste generated as a result of the industrial revolution between 16th and 18th centuries (Wilson, 1977; Petts and Eduljee, 1994; Williams, 2005). In the developed countries the management of waste at all stages of production, storage, processing, treatment, and ultimate disposal, has recently become a social and political imperative that has led to a changing perspective in management. The new perspective is that waste should be recovered or disposed of without risks to human health, and provision of appropriate treatment options to minimise environmental impacts; particularly without risk to water, air, soil, plants and animals (Petts and Eduljee, 1994; Directive 75/442/EEC).

1.2.2 Waste Disposal and Treatment options

A key factor in selecting any waste management option for a particular waste is the provision of appropriate treatment and, or disposal facilities which can provide the best environmental option alongside the principle of sustainable development (Petts and Eduljee, 1994; Williams, 2005). Other factors which must be taken into consideration are: capital investment costs of the facility, operating and maintenance costs, and proximity to the source of waste arising, *i.e.* waste should be disposed of or dealt with close to the place where it arises (Williams, 2005). The broad meaning of what constitutes 'disposal' is provided in Annex II A of the Waste Framework Directive (Directive 74/442/EEC). However, in the context which it is used in this thesis, discussion of the term 'disposal' is limited to the practice of landfilling. Treatment process options can be classified into four basic types (Petts and Eduljee, 1994; Directive 99/31/EC).

- Physical
- Chemical
- Thermal
- Biological

Treatment processes are aimed at stabilising the waste, reducing or eliminating their intrinsic toxicity, thus lowering their impact on the environment. In some cases treatment converts waste

to useful products (Petts and Eduljee, 1994; Cheremisinoff, 2003). A few examples of specific processes within each of the treatment categories are given on Table 1.4.

Table 1.4: Waste treatment options (Petts and Eduljee, 1994).

Alternative	Brief description	Example application
A. Physical treatment		
A1. Sedimentation	Gravity settling of particles suspended in liquid.	Used by sewage treatment plants.
A2. Screening	Removes large particles from wastewater.	Wastewater containing solids etc.
B. Chemical treatment		
B1. Oxidation/reduction	Wastes made less toxic, simpler & less volatile by subtracting or adding electrons between reactants.	For inorganic/organic wastes, e.g. aromatic hydrocarbons, sulfur compounds, cyanides etc.
B2. Neutralisation	Mixing acid or alkaline wastes with buffering agent to produce solution (pH = 7.0)	Acid pickle liquor from metal cleaning; spent acid catalysts; tanning wastes etc.
C. Biological treatment	Uses living micro-organisms to decompose organic waste into water, CO_2 and simple inorganics or simpler organics such as aldehydes and acids.	For biodegradation of waste.
C1. Composting	'Engineered' mounds of waste to encourage biological breakdown of organic solids so as to stabilise & sanitise them, producing a humic substance valuable as a soil conditioner.	Domestic refuse (often source separated)
C2. Anaerobic digestion	Decomposing organic matter in a closed vessel in the absence of air. Uses two forms of bacteria: acid-forming and methane-forming.	Considered suitable for simple organics typically found in municipal wastewaters. For treating sludge containing 5-7% solids.
D. Thermal treatment		
D1. Incineration	Enclosed device using flame. Temperature range 850°C-1200°C.	Combustible solids, sludge, liquids, municipal refuse.
D2. Pyrolysis	Destructive distillation in absence of oxygen. Wastes broken down into solids, liquid and gaseous components.	Organic solids.

1.2.3 Waste management hierarchy

Minimisation of waste is the top priority of the waste management hierarchy, and it is one of the guiding principals for solid waste management throughout the developed world (Read *et al.*, 1997). In the European Union, the national strategy for treatment and disposal of waste has been developed through a series of legislative directives and policies which has the main objectives of resource recovery and environmentally sound disposal approaches (Directive 75/442/EEC; COM 2005).

The Waste Framework Directive of 1975 (Directive 75/442/EEC) is the most important Directive concerning waste management in the European Union. The Directive defines 'waste', as 'any substance, or object listed in the Directive, which the holder discards or intends or is required to discard'. Although it has since been amended many times, including 2008 (Directive 2008/98/EC) it introduced the principle of the hierarchy of waste management (Figure 1.3) and set out key objectives that waste should be prevented, and what cannot be prevented should be re-used, recycled, and recovered, with disposal (landfill) being the least preferred option.

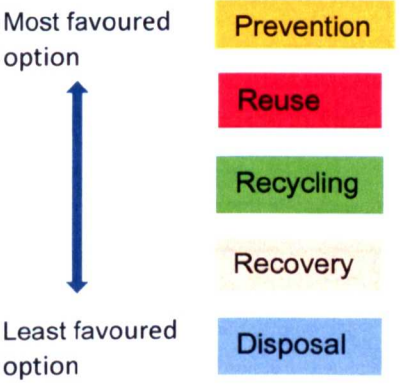

Figure 1.3: An illustration of the waste hierarchy based on Waste Framework Directive 2008/98/EC.

The current European Union's policy on waste management is based on this concept of a hierarchy of options, where the most desirable is not to produce the waste in the first place, *i.e.* prevention and reduction through clean and improved technologies, re-use and recycling, recovery, and optimisation of final disposal that provides the most benefits or least damage to the environment (COM, 2005).

As part of a waste management strategy to recover materials composting, anaerobic digestion, and incineration with energy recovery are part of treatment options considered in this hierarchy.

Waste is increasingly being considered as a valuable resource to the industry and a major economic sector (COM, 2005). From the economic perspective the drive to deal with waste in environmentally sound ways has created jobs and business opportunities in Europe. The sector has a high growth rate with an estimated turnover of over € 100 billion and provides about 1.2 to 1.5 million jobs (COM, 2005).

1.2.4 Waste generation

The combined waste generation in the European Union is estimated at 1.3 billion tonnes per year (Eurostat 2005). Industrial solid wastes, including sludge, form a major part of the EU's solid waste management problems. Table 1.5 gives the latest statistical distribution of waste generated by major economic sectors (EC, 2001; Eurostat 2005). The leather industry, as a sub-sector of the manufacturing industry, is covered in that category. The variable composition of waste from the manufacturing industry included hazardous, non-hazardous and biodegradable waste (Eurostat, 2005).

Table 1.5: Estimated annual waste generated in the European Union by sector (Eurostat, 2005).

Sector	Million tonnes
Construction	510
Manufacturing industry	427
Energy and water supply	127
Municipal waste	241

1.2.4.1 Current disposal and treatment practices

Figure 1.4 represents a summary of the percentage volume of waste treated by the main disposal and treatment types in the European Community. Although landfilling is the least preferred option according to the waste hierarchy, it is still the most used method of disposal, with 49% of the waste being consigned to landfill. However, it is also apparent that resource

recovery has been entrenched as a fundamental component of waste management strategies in the European Community, with 33% of the waste being recycled and composted (COM, 2005).

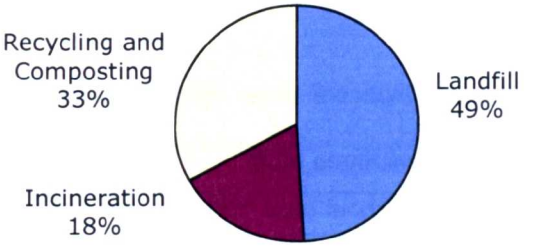

Figure 1.4: Main waste disposal and treatment methods in the EU (COM 2005).

1.2.5 Biodegradable waste

Biodegradable waste is the type of waste that can undergo anaerobic (without oxygen) or aerobic (in presence of oxygen) decomposition (Alison *et al.*, 2001). It is the type of waste that is biologically degraded in the landfill by micro-organisms producing gases and liquid leachates (Williams, 2005).

1.2.6 Definition of Landfill

'Landfill' is defined in the EU Landfill Directive 99/31/EC (1999) as 'a waste disposal site for the deposit of the waste onto or into land (*i.e.* underground), including:

- Internal waste disposal site where a producer of waste is carrying out its own waste disposal at the place of production.
- Site used for more than one year for temporary storage of waste.

1.2.6.1 Environmental impact of the main disposal options

Table 1.6 is a summary of some of the environmental impacts of the main waste treatment options.

Table 1.6: Some environmental impact of main waste management options (Alison *et al.*, 2001).

Option	Main environmental impacts
Landfill	▪ Methane emission from biodegradable waste, contributing to global warming and local hazards such as risk of fires ▪ Risk of water pollution from leachate formed as waste decomposes and odour. ▪ Land use: non-sustainable use of resources. ▪ Long retention of some carbon compounds.
Incineration	▪ Emission of harmful airborne pollutants such as NO_x, SO_2, HCl, fine particulates and dioxin. ▪ Emission of CO_2 from fossil-derived waste (e.g. plastics), N_2O contributing to global warming. ▪ Fly ash and residues from air pollution control systems require stabilisation and disposal as hazardous waste.
Composting	▪ Avoids methane production as degradation is aerobic. ▪ Can be used as a soil improver to replace inorganic fertiliser ▪ Process needs careful control to avoid bio-aerosols.
Anaerobic digestion	▪ As composting: plus energy recovered can replace fossil fuels thus avoiding emissions of CO_2.
Recycling	▪ Saves energy (less energy required to manufacture products from recycled feedstock), no emissions. ▪ Contributes to sustainable use of resources.
Mechanical biological treatment (MBT).	▪ Reduces methane and liquid leachate production. ▪ Still dependent on landfill as final repository of final waste, so not as sustainable as composting or recycling. ▪ Materials recovered for recycling and/or energy recovery.

1.2.7 Environmental Legislation relevant to the tanning industry

There are no specific Directives for the tanning sector but several Directives have implications for the industry, particularly regarding its impact on human health, and the environment. In addition to the key legislation covered in Waste Framework Directive of 1975 (74/442/EEC), the

other Directives that drive the agenda for pollution and waste minimisation in the leather industry are as follows:

1.2.7.1 Landfill Directive 99/31/EC

Directive 99/31/EC of 16th July 1999, concerning landfilling, is the legislation driving the need to divert biodegradable solid waste from landfill. The rationale behind the legislation is based on the concerns over pollution of surface and groundwater, and the effects of greenhouse gas emissions and the threat to the environment as well as human health. Hence, the Directive imposes a reduction in the amount of biodegradable solid waste disposed in landfill while seeking to enhance waste minimisation through increased level of recycling and recovery. The Directive has set demanding reduction targets to all EU member states as follows:

- By 2010 to reduce biodegradable municipal waste landfilled to 75% of that produced in 1995.
- By 2013 to reduce biodegradable municipal waste landfilled to 50% of that produced in 1995.
- By 2020 to reduce biodegradable municipal waste landfilled to 35% of that produced in 1995.

1.2.7.2 Integrated Pollution Prevention and Control (IPPC) Directive 96/61/EC

The IPPC Directive 96/61/EC of 24th September 1996 is the main EU legislation that directly affects the leather industry. The overall objective of the Directive is to prevent or minimise water, soil, and air pollution by emissions arising from industrial processes to achieve environmental sustainability. Through its technical bureau, the Commission has produced a series of 'Reference Documents' (BREFs) which describes the most suitable environmental alternative ways of operating processes for each sector based on the concept of 'best available techniques' (BAT). A broad definition of BAT has been given in Article 2(11) of the Directive. The overall objective of BAT is to provide guidance on the most effective practical operating techniques designed to prevent emissions, and where that is not possible, to reduce emissions and the general impact on the environment.

The Directive demands that all tanneries, where the treatment capacity exceeds 12 tonnes of finished products per day, operate according to an integrated permitting conditions based on 'best available techniques' (BATs) published by the Commission (IPPC, 2003).

1.2.7.3 The Sewage Sludge Directive 86/278/EEC

Directive 86/278/EEC of 12th June 1986 on the protection of the soil seeks to encourage the use of sewage sludge from treatment plants in agriculture, and provides regulatory limit values for concentration of heavy metals so as to prevent harmful effects on soil, vegetation, animals, and man. The Directive defines treated sludge as that which has undergone 'biological, chemical, heat treatment, or any other appropriate process so as to reduce its fermentability and the health hazards resulting from its use. The Directive also requires that sludge should be used in such a way that take into account the nutrient requirements of plants, and that the quality of the soil, surface and groundwater are not impaired. The waste sludge from the tannery effluent treatment plants are covered by this legislation.

1.2.7.4 Animal By-Products Regulation (ABPR) 1774/2002

The Animal by-products Regulation (ABPR) 1774/2002 of 3rd October 2002, provides guidelines on the efficient management and treatment of animal by-products not intended for human consumption. Raw materials such as hides and skins, which are used outside the food chain for leather processing, are covered under this regulation.

1.2.8 Impact of Best Available Techniques on the Tanning Industry

The BAT recommendations prepared for the leather industry in response to the IPPC Directive 96/61/EC (IPPC, 2003), enabled the tanning industry to identify the key environmental challenges for the sector, identification of the techniques most relevant to address those challenges and the best environmental performance levels based on the available data in the European Union and worldwide. The key areas of environmental concern for the tanneries are wastewater, solid waste, air pollution, soil (land) protection and health and safety aspects (IPPC, 2003). On the basis of this assessment, detailed process-integrated measures and methodologies to improve the wastewater quality and minimise environmental impact were proposed. The proposed adjustments included reduction of water consumption, replacement of

harmful process reagents, and provisions for waste recovery for recycling and reuse. Towards meeting these objectives the reduction of the use of sulfide during unhairing and hair recovery by the use of enzyme preparations are considered as a priority with respect to reducing chemical and biological oxygen demands, odour, nitrogen, and toxicity parameters (IPPC, 2003). Consequently, a number of hair-save (hair recovery) unhairing techniques have been developed and are routinely practiced (Frendrup, 2000).

In search of a sustainable and efficient utilisation of the recovered hair it is necessary to have detailed knowledge of its general physical properties and chemical composition.

1.3 Hair: morphology, composition and biochemical structure

1.3.1 General properties of hair

Hair is a characteristic extracellular appendage of the epidermis of mammals. It acts as a protective barrier and plays an important part in the adaptation of vertebrates to their environmental conditions. Its chemical composition is closely allied to other integument appendages such as wool, feathers, nails, horns and hooves (Mathews, 1954; Fraser *et al.*, 1972). Mammalian hair is proteinaceous, consisting predominantly of the class known as keratin, which makes up 1-2% of the skin weight. The word keratin is derived from the Greek word meaning 'horn' (Fraser *et al.*, 1972; Sharphouse, 1989; Bienkiewicz, 1983). During the industrial leather manufacturing process hair and other epidermal substances are removed, and along with lime and sulfide constitutes the biggest contaminant in the effluent (Sharphouse, 1989).

Keratin belongs to the family of structural proteins known as intermediate filaments (IF), and are well known for their insolubility and resistance to chemical and enzymatic digestion. The stability is mainly due to the presence of numerous disulfide bonds and other crosslinks such as hydrogen bonds, salt linkages and hydrophobic interactions (Mathews, 1954; Fraser *et al.*, 1972; Jones, 1976; Feughelman, 2002).

Keratins are subdivided into two groups: 'hard and soft' keratins according to the degree of disulfide bonds. Hard keratins are found in hair, nails and hooves and have a high content of disulfide bonds. Therefore, hard keratins are tough and more resistant to degradation. On the

other hand, soft keratins found in *stratum corneum*, corns, and callouses have low content of disulfide bonds and are more pliable (Fraser *et al.*, 1972; Gupta and Ramnani, 2006). Keratin and keratin-containing tissues are further classified according to their x-ray conformation structure as: α-keratin (α-helix) in hard keratins and β-keratin (β-sheet) in feathers and other avian species (Fraser *et al.*, 1972; Bradbury, 1973).

1.3.2 Morphology and chemical composition of hair

1.3.2.1 Hair growth: the hair follicle

Hair growth is initiated by cellular events within specialised cells of the dermis leading to the development of the follicle. The formation of the hair follicle begins by means of division of the cells around the dermal papilla (Figure 1.5). The dermal papilla, which play a crucial role in the regulation of successive cycles of hair growth, remains attached to the basal plate as the major connective tissue as further cell differentiation takes place towards the epidermis giving rise to the inner root sheath (Jahoda and Reynolds, 1993; Montagna and Parakkal, 1974). Further cell division in the follicle matrix brings about the development of other major structural components such as the medulla, cortex, hair cuticle, the huxley layer, and Henley layer (Birbeck and Mercer, 1957; Montagna and Parakkal, 1974; Krause and Foitzik, 2006). The bulbous part (dermal papilla) of the follicle contains the most mitotically active cells, the keratinocytes of the hair matrix and melanin granules (Montagna *et al.*, 1964; Krause and Foitzik, 2006). As the cells differentiate upwards, there is an increase in the fibrils and the bulb begins to constrict at the pre-keratinised (soft keratin) region. Above the pre-keratinised zone there is the keratogenous zone, the fibrils aggregate into macrofibrils and become keratinised (hardened). Progressive hardening (keratinisation) proceeds in the hair shaft through the formation of disulfide linkages, conferring stability to the structure. The mature hair follicle is a complex structure (Figure 1.5) composed of concentric cylinders of epithelial cells, known as root sheaths, surrounding the cylindrical hair shaft in its centre (Figure 1.5) (Montagna *et al.*, 1964; Montagna and Parakkal, 1974; Krause and Foitzik, 2006).

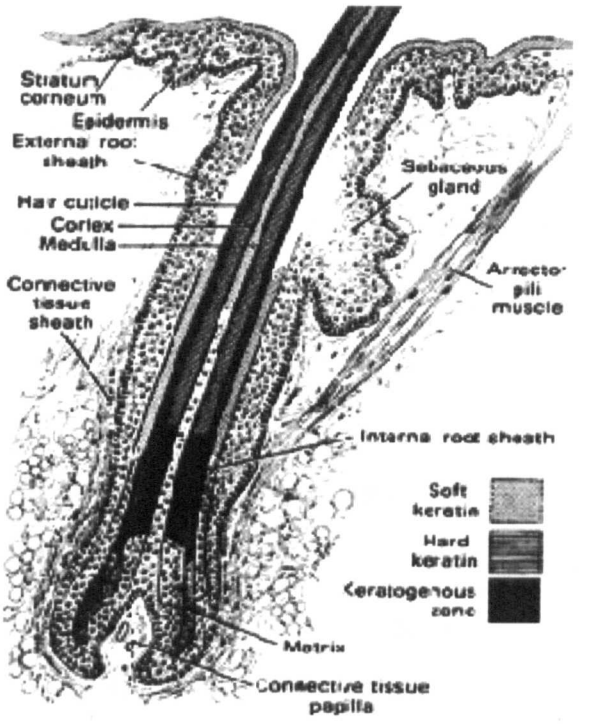

Figure 1.5: An illustration of medullated mature hair follicle showing different levels of keratinisation: the keratogenous zone, hard keratin and soft keratin (EL Baba *et al.*, 2000).

The growth of the hair follicle is not continuous but undergoes cycles of renewal in three main phases known as anagen, catagen and telogen (Montagna and Parakkal, 1974; Krause and Foitzik, 2006). Anagen is the active growing phase where the bulbous matrix cells give rise to all the cells of the hair shaft and pigmentation of the cortical cells occurs. Catagen, the transition phase, is characterised by morphological changes and controlled reorganisation of the follicle. Active growth and melanin production ceases as the follicle shrinks and the dermal papilla condenses. The final resting phase, the telogen, is marked by complete biochemical inactivity and the dermal papilla becomes detached from the follicle (Montagna and Parakkal, 1974; Krause and Foitzik, 2006; Garro *et al.*, 2006).

1.3.2.2 Chemical composition of hair

The chemical composition of mammalian hair varies with its water content, but the main component is protein, making 65-95% of the hair weight. The remaining constituents are water, lipids, pigment, and trace elements. The five key elements present in hair are carbon, hydrogen, nitrogen, oxygen, and sulfur (Mathews, 1954; Dawber, 1996). A fundamental feature of the chemical composition hair proteins is the presence of the disulfide (S–S) amino acid, cystine, in

between the polypeptide chains (Figure 1.6). Cystine is the main constituent amino acid in keratin (Fraser *et al.*, 1972; Feughelman, 2002). The formation of cystine crosslinks is as a result of oxidation of the sulfurhydryl groups of the amino acid, methionine. The presence of these disulfide crosslinks allows the formation of a three dimensional structure, thereby conferring stability and insolubility in the common protein solvents (Feughelman, 2002).

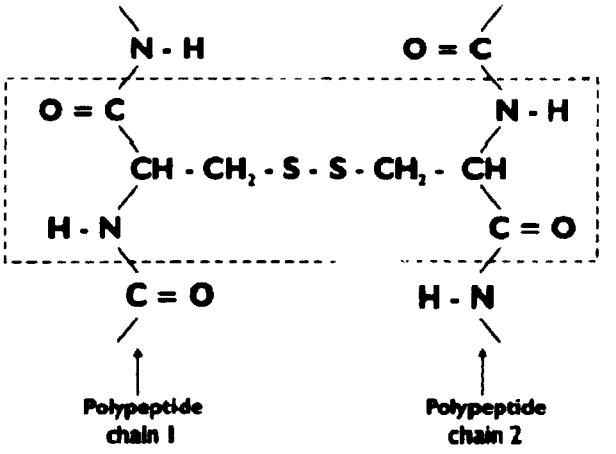

Figure 1.6: Representation of the cystine disulfide crosslinks between polypeptides (Feughelman, 2002).

While keratin constitutes the main protein of hair, it is also known to contain a sizeable fraction of non-keratinous proteins as well as non-proteinaceous material. The non-keratinous proteins may be defined as those which are only slightly crosslinked (low-sulfur proteins) and are easily degraded, both hydrolytically and enzymatically. They are found embedded as a network in the keratinous protein yielding a biological 'composite' structure. The non-keratinous proteins generally provide chemical and physical weak spots in keratinous tissues (Mercer, 1953; Bauman, 1979; Bienkeiwicz, 1983). Present within the inter-microfibrillar matrix of the hard α-keratin are the high-sulfur and high-glycine-tyrosine proteins (Fraser *et al.*, 1972).

Mammalian hair keratin protein exhibits amphoteric properties (containing both cationic and anionic groups). The cationic property is the result of the protonated side chains of arginine, lysine and histidine, whilst the anionic character is due to acidic side chains of glutamic and aspartic acids (Mathews, 1954).

1.3.2.3 Biochemical structure of hair

Mammalian hair has a complex structure that comprises of three common histological features: (1) the cuticle, (2) cortex, and (3) medulla (may be present only in coarse keratin fibres). Figure 1.7 is a representation of the major structural components of hair (Stoves, 1948; Blackburn, 1948; Jones, 2001).

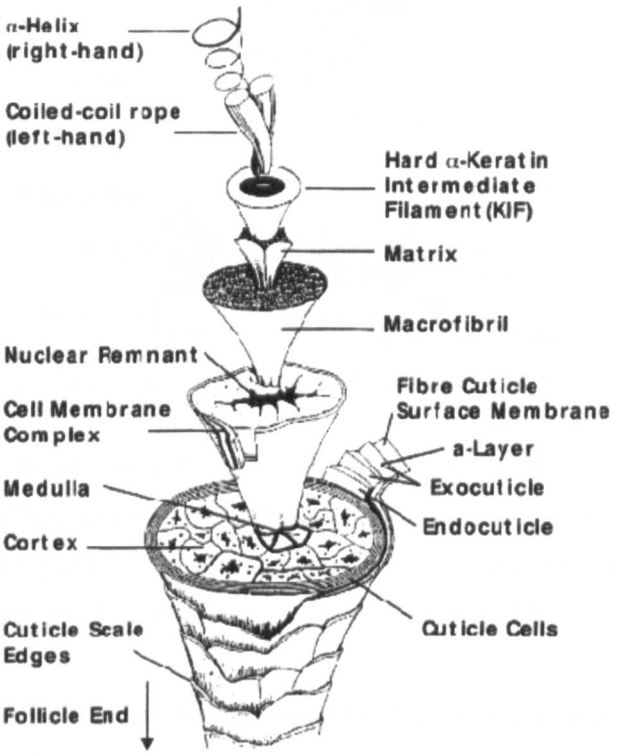

Figure 1.7: Schematic diagram showing major structural components of hair shaft (Jones, 2001).

The biochemical and chemical susceptibility of individual keratin fibres is known to be highly dependent on these basic structural components, their chemical composition, and degree of disulfide crosslinks. The stability increases with increasing crosslink density (Addy *et al.*, 2001; Bauman, 1979; El Baba *et al.,* 2000; Stoves, 1948). The external surface of the fibre is covered by the scaly cuticle layer, which is made up of flattened overlapping cells. Each cuticle cell exists as a composite of lamellar components (Figures 1.7 & 1.8) containing two major intracellular layers: the outer sulfur-rich band known as the exocuticle (consists of A-layer and epicuticle), and the inner region of low-sulfur proteins called the endocuticle (Dawber, 1996; Orwin, 1979; Swift and Smith, 2001). Each cell is separated from each other by an intracellular

membrane, cell membrane complex (CMC), which also acts as an adhesive to the individual cells onto the underlying cortex. The CMC comprises of bilayer components of the less crosslinked δ-layer on the inner side and the hydrophobic lipid-rich β-layer on the outside (Figure 1.8) (Swift and Smith, 2001; Jones, 2001).

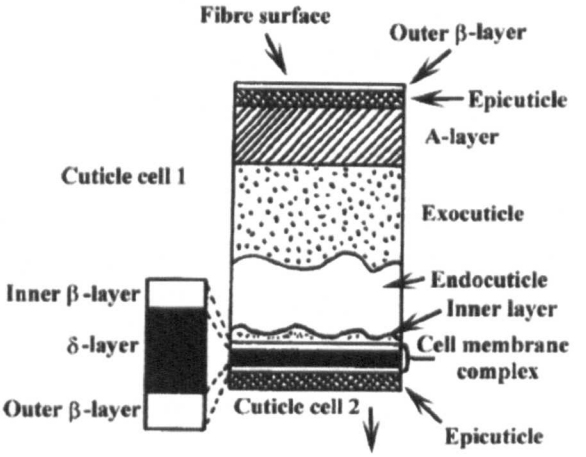

Figure 1.8: Schematic diagram of cross-section of the hair cuticle (Swift and Smith, 2001).

Cuticle provides protection to the internal components of the fibre, and due to its densely crosslinked nature it is highly resistant to chemical and enzymatic degradation. Its surface also acts as a tough diffusion barrier due to the hydrophobic properties of the tough cementing external lipid layer (Cubarsi *et al.*, 2006; Jones and Rivett, 1997; Mercer, 1953; Stoves, 1948).

The cortex constitutes the bulk of the hair, making 85-90% of the total hair protein. It is the part that is responsible for the fibre's mechanical properties and intrinsic strength (Jones and Rivett, 1997; Mathews, 1954). The cells of the cortex are elongated, spindle-shaped, fused tightly and oriented parallel to the fibre axis. Each cell is composed of axially oriented microfibrils of α-keratins which group into larger units forming macrofibrils. The macrofibrils represent about 50 to 60% of the cortex material, by mass. Other constituents are melanin granules, cytoplasmic remnants, and intercellular membrane (Fraser *et al.*, 1972; Jones and Rivett, 1997; Wolfram, 2003). The cells within the cortical macrofibrils are classified into two groups according to their degree of disulfide crosslinks. They are respectively designated as paracortex and orthocortex. The para components of the fibre are densely crosslinked, having more sulfur proteins than the ortho components which contain low sulfur proteins, and are surrounded by more of the intermacrofibrillar material consisting of non-keratinous proteins (Dawber 1996; Fraser *et al.*, 1972; Manich *et al.*, 2005; Orwin, 1979).

The medulla forms the central core of the fibre, and it is present only in coarse keratin fibres. Like other structural components medullary cells originate from the bulb and are present as vacuolated cells in the mature tissue. The vacuoles and other cellular spaces become air-filled thus increasing the stability and light-reflection properties of the fibre (Mathews, 1954; Orwin, 1979). Medullary cells contain a large amount of protein citrulline, and an appreciable amount of glutamic acid and glutamine. Cystine, the amino acid responsible for the stabilisation of the fibrous keratins is, however, absent in the medulla. The cells are believed to be stabilised via the ε-(γ-glutamyl)-lysine linkages (Montagna and Parakkal, 1974; Orwin, 1979; Rogers, 1962; Stoves, 1948). The medullary proteins are usually insoluble in alkaline treatment and resistant to other keratinolytic agents. However, the proteins can be degraded by proteolytic enzymes (Blackburn, 1948; Stoves, 1948; Fraser *et al.*, 1972).

1.3.2.4 Mechanism of hair degradation

Hair can be destroyed, or disintegrated by the action of alkali and reducing agent. In practice the alkali used is calcium hydroxide (lime), sometimes with the addition of sodium hydroxide or sodium carbonate. The reducing agent generally used is sodium sulfide (Merill, 1956; Morris, 1956). A number of reviews on the subject of chemical and biochemical destruction of hair have been published (Addy *et al.*, 2001; El Baba *et al.*, 2000; Germann, 1997). The mechanism of hair destruction operates on the basis of differences in its structural components, and it has been suggested to comprise of the following steps (Addy *et al.*, 2001; El Baba *et al.*, 2000):

- Sulfide attacks the hair from the tip, where the inner structures have been exposed due to the weathering effect following the continued action of heat, light and water (popularly known as 'split ends').
- The reaction proceeds downward through the cortex where the cystine disulfide links are broken by the sulfide ion causing the fibre to breakdown and go into solution.
- The medulla is attacked by the hydrolysing effect of the hydroxyl ions (OH^-), from the effect of the lime (calcium hydroxide) at pH 12.5. However, if a proteolytic enzyme is present, the degradation rates of both the medulla and the cortex may be enhanced.
- The cuticle is not dissolved in the time scale of the industrial process, but is mechanically broken up.

Due to their constituent highly crosslinked proteins, the cortex and the cuticle control the rate of hair destruction (Merril, 1956). It is assumed that during the industrial unhairing processes with hair recovery, through the hair-save technology, the integrity of the hair shaft is retained, since hair is attacked at its attachment point at the base of the follicle (Addy *et al.*, 2001; El Baba *et al.*, 2000; Germann, 1997). This suggests that if the recovered hair were to undergo biodegradation, the biodegradation process would occur on the cuticle surface since the more vulnerable components are far less accessible for attack. However, it is known that hair is generally highly resistant to biodegradation, and may, therefore, require some form of pretreatment to enhance the biodegradation process.

1.3.2.5 Melanin in hair

The word 'melanin' is derived from the Greek word meaning black, but it refers to a range of naturally occurring pigments widely distributed among living organisms, including higher plants, fungi, and bacteria (Lerner and Fitzpatrick, 1950; Prota, 1980). It is the predominant pigment observed on the mammalian skins, hair, and feathers, but can also be found in other locations like eyes, brain, and inner ear. The pigments are synthesised in specialised cell organelles, premelanosomes and melanosomes, within the pigment producing cells, melanocytes (Chakraborty and Roy, 2003; Jimbow *et al.*, 1976; Orlow, 1995; Prota, 1980). Melanosomes are transferred in skin via keratinocytes, and in the hair bulb for final distribution into the differentiating cortical cells (Hearing and Tsukamoto, 1991; Parakkal, 1967).

Structurally melanin exists as an amorphous, insoluble, and heterogeneous indolic biopolymer. The biogenesis in the melanocytes is initiated by the hydroxylation of the amino acid tyrosine to L-dopa (3,4-dihydroxyphenylalanine) followed by oxidation of the L-dopa to Dopaquinone (3,4-dioxyphenylalanine). Both steps of the reactions are catalysed by the copper-containing enzyme, tyrosinase (Duliere and Raper, 1930; Hearing and Ekel, 1976; Raper, 1926; Riley, 1997). Dopaquinone then undergoes spontaneous cyclisation and polymerisation to yield 5-6-dihydroxyindole-2-carboxylic acid (DHIC), the insoluble black melanin (Meredith and Sarna, 2006; Ortonne and Prota, 1993; Prota, 1980). Alternatively, other compounds may take part in polymer formation leading to different structures, e.g. nucleophilic addition of cysteine to dopaquinone leading to the formation of cysteinyldopa and benzothiazine derivatives. These products are formed via intramolecular cyclisation of thiol conjugates of dopaquinone (Borges *et al.*, 2001; Ito and Wakamtsu, 2008; Jimbow *et al.*, 1976; Ortonne and Prota, 1993). Whereas

plants characteristically express catechol melanins, mammalian melanins are generally composed of indole-5-6-quinone units. However, the metabolic formation of melanin in all living organisms follows a similar mechanism catalysed by the enzyme tyrosinase (Jimbow *et al.*, 1976; Prota, 1980).

There are two main classes of animal melanins, grouped according to colour and solubility properties: eumelanin (black-brown) and pheomelanin (yellow-red) (Figure 1.9) (Ito and Jimbow, 1983; Ito and Wakamatsu, 2008; Prota, 1980).

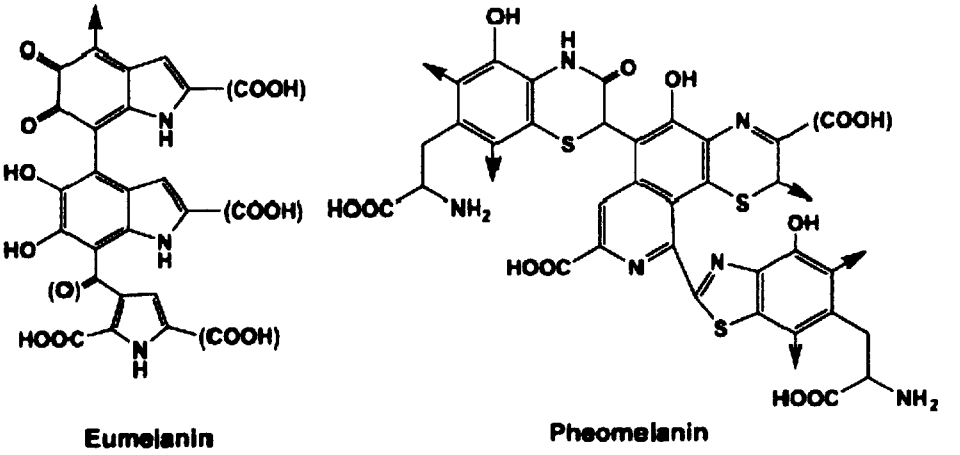

Figure 1.9: Structures of eumelanin and pheomelanin (Ito and Wakamatsu, 2008).

Eumelanins are insoluble in acid and alkali; contain 6-9% nitrogen, and 0-1% sulfur. Pheomelanins are soluble in alkali, contains 8-11% nitrogen, and 9-12% sulfur (Ito and Jimbow, 1983). Eumelanins are the products of the oxidative copolymerisation of 5,6-dihroxyindole and 5,6-dihydroxindole-2-carboxylic acid units, whereas pheomelanins contain 1,4-benzothiazine units derived from 2-cysteinyldopas (Ortonne and Prota, 1993; Borges *et al.*, 2001; Donato and Napolitano 2003). Figure 1.10 is a representation of the biosynthetic pathway of the two types of melanins according to Ito and Wakamatsu (2008).

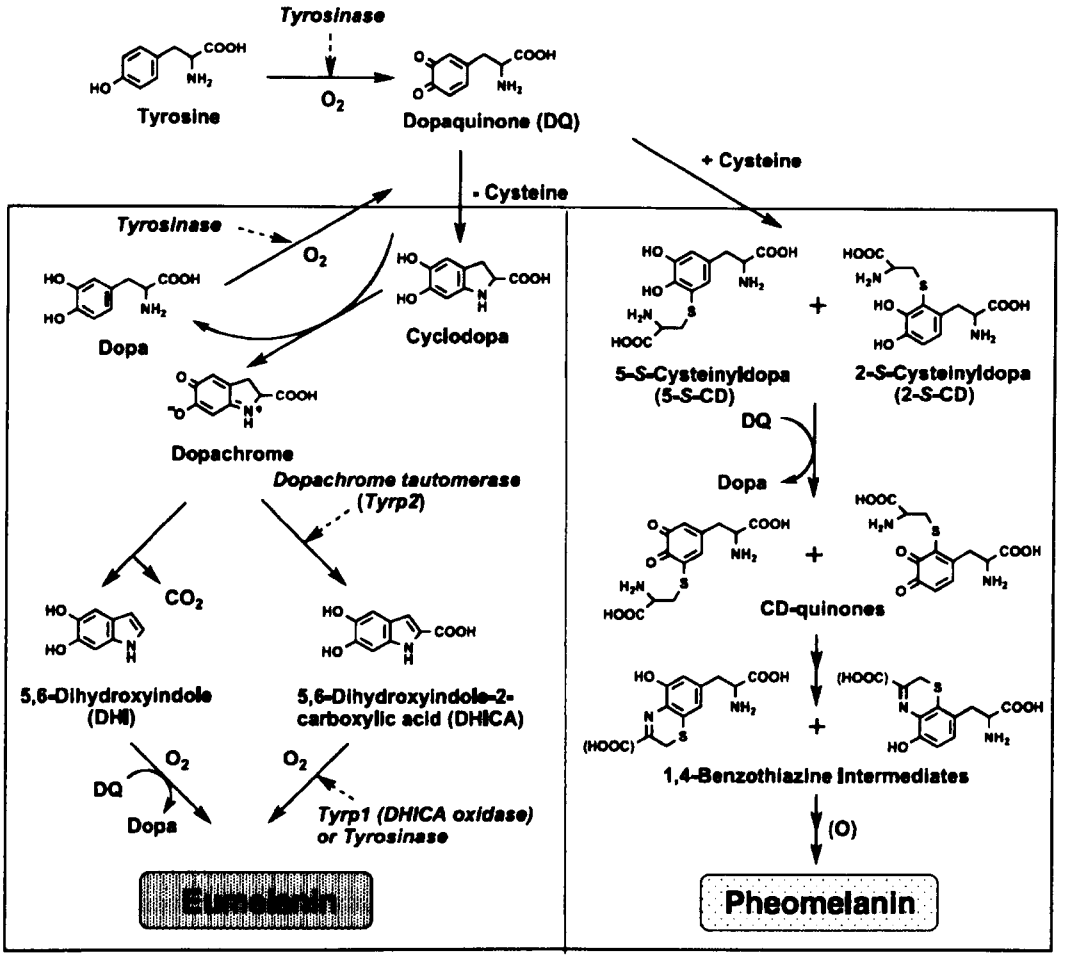

Figure 1.10: Biosynthetic pathways leading to eumelanin and pheomelanin production (Ito and Wakamatsu, 2008).

Melanin is always associated with proteins initially present as premelanosomes, which acts as either scaffolding matrix for melanin deposition, or as enzymes catalysing the various steps of the oxidation process, and initialising polymerisation (Wagh *et al.*, 2000). Although it appears that the binding of melanin with protein is an essential aspect of melanin polymer formation there is conflicting data regarding the type of binding and interactions involved (Whittaker, 1979; Mani *et al.*, 2001). However, it has been suggested that charge interaction plays an important role in the melano-protein complex. This arises from the fact that melanin is a negatively charged polymer due to its numerous COO^- (carboxyl) groups (Mani *et al.*, 2001).

The biological function of melanin is to provide photochemical protection to the tissue against a broad band of ultraviolet (UV). Other functions include thermoregulation, free-radical scavenging to relieve cells of potential oxidation stresses, and chelation of toxic heavy metals (Riley, 1997;

Kevin, 2005; Claus and Decker, 2006; Meredith and Sarna, 2006). Other observations have been made with regard to its ability to provide protection against microbial and enzymatic degradation in plants, fungi (Claus and Decker, 2006; Yuan *et al.*, 2007), and more recently, feathers (Goldstein *et al.*, 2004). It has also been associated with protein crosslinking leading to enhanced mechanical properties of some biological tissues, sclerotisation of insect cuticles, and strengthening of plant cell walls (Bonser, 1995; Riley, 1997; Goldstein *et al.*, 2004; Yuan *et al.*, 2007). In animal hair, melanin granules fill the cortical cells and are tightly held within a keratin matrix of the cortex (Mathews, 1954; Cubarsi *et al.*, 2006; Liu and Simon, 2003; Liu *et al.* 2003). Apart from its photochemical protection the natural role of melanin in hair is not known (Borges *et al.*, 2001).

In the leather industry melanin is encountered during the tanning of different types of raw hides, typically black, brown, and reddish-brown bovine hides or calfskins. It is normally observed as retained patchy pigmentations on the surface of the raw material after unhairing and liming process (Punitha *et al.* 2008). There have been unpublished claims and unconfirmed belief in the tanning industry that during the conventional chemical unhairing process using sodium sulfide and lime (calcium hydroxide), the solubilisation of black and brown hair is slower than the white hair under typical hair dissolving conditions. This might, of course, be due to the difference in ease of observing pigmented versus non-pigmented hairs, although the claims indicate there is some effect (Covington, 2006).

1.3.2.6 Principles of Hair-save

Section 1.1.4.2, on beamhouse process of liming and unhairing, introduced the two technologies of hair removal that may be adopted during the leather manufacturing process (*i.e.* hair recovery and hair destruction methods). The unhairing process by hair destruction using lime and sodium sulfide is a practice that has generally been adopted in many countries because it is fast, cleanses and opens up the hide substance to a satisfactory degree (Frendrup, 2000). However, it is by far the largest single source of organic pollution and suspended solids in tannery wastewater (Frendrup, 2000; El Baba *et al.*, 1999; Happich *et al.*, 1974). Moreover, due to stringent environmental regulations, treating the wastewater has become very expensive and results in a large amount of sludge posing disposal problems (Frendrup, 2000).

The principles of hair-save technologies involve manipulating the chemistry of the mechanism of unhairing, chemical properties of hair, and alkalinity of the system to cause hair to be dislodged from its follicle, and therefore, be mechanically removed with the hair shaft intact (Germann, 1997; El Baba *et al.*, 2000, Frendrup, 2000). Almost in all methods, the fundamental principle entails immunising the hair (*i.e.* controlled pretreatment with lime to protect the hair from the reducing action of sulfide). This is followed by a chemical attack on the pre-keratinised zone of the hair bulb and/or enzymatic digestion of the connective tissues of the epithelial cells surrounding the hair follicle (Figure 1.5), and collagenous proteins of the basement membrane causing hair to weaken at its attachment base (El Baba *et al.*, 2000; Addy *et al.*, 2001). The lime-pretreatment (immunisation) changes the chemical character of hair by converting the cystine in hair to a new amino acid residue known as lanthionine, which is resistant to cleavage by sulfide (Bienkeiwicz, 1983; Windus and Showell, 1968)

The typical hair-save technologies are based on three main methods (Cantera *et al.*, 1995; Frendrup, 2000):

(i) Lime-controlled pretreatment of hair (hair protection, *i.e.* immunisation) followed by unhairing based on sodium sulfide (decrease in the amount added).

(ii) Lime-controlled pretreatment of hair (immunisation) followed by unhairing based on sodium sulfide (reduced amount added) supported by commercial products based on organic sulfur compounds (mercaptans and thiols), and amines.

(iii) Lime-controlled pretreatment of hair (immunisation) followed by unhairing based on sodium sulfide (reduced amount added) supported by the use of enzymatic products with proteolytic activity, in the presence of lime.

1.3.2.7 Environmental impact of hair-save unhairing processes

The development of the hair-save unhairing processes with hair recovery has enabled a number of tanneries to realise environmental benefits, with regard to the level of pollution of effluent originating from the beamhouse processes. This includes decreases in the level of organic load in the wastewaters in terms of chemical oxygen demand (40 – 60%), biological oxygen demand (50%), sulfide content (50%), and suspended solids (60%) (Cantera *et al.*, 1995; IPPC, 2003; White *et al.*, 1991). The amount of hair recovered through the hair-save processes varies from 30 – 50 kg dry hair per tonne of raw bovine hide and up to more than 100 kg per tonne of calf, sheep or goat skins (IPPC, 2003). There have also been reported cases of a possible recovery

of up to 30 – 40 tonnes of hair waste per week from a tannery processing 10,000 hides per week using the hair-save technology (White *et al.*, 1991).

Although environmental benefits of hair recovery have been realised within the tanning industry, however, the recovered hair presents some environmental and economic concerns. The accumulation of the recovered hair constitutes a substantial source of solid waste that would normally be disposed in landfill, which is one of the major environmental concerns (IPPC, 2003; Frendrup, 2000; Directive 99/31/EC). Consequently, the tanners have to incur disposal costs in order to fulfil environmental legislative requirements. EU tanners' environmental costs, for example, are estimated at about 5% of their turnover (IPPC, 2003). Therefore, an alternative viable treatment of the recovered hair would be attractive to the tanning industry.

1.3.2.8 Utilisation of hair waste

A number of possible applications for the recovered hair have been suggested based on its physical and chemical properties. These include using hair as a felting material, protein supplement in animal feeds, personal care products, biogas production, and more recently as a biomaterial (Barba *et al.*, 2008; Cantera and Buljan, 1997; Money, 1996; Sierpinski *et al.*, 2008). The greatest potential use of the recovered hair is widely believed to be as a nutrient source, of slow-release nitrogen, for agricultural purposes. This view is derived from the fact that hair contains 15% nitrogen, dry weight (Onyuka, 2010). The treatment of tannery hair waste through composting to produce a product that can be used as organic fertiliser has been suggested to be the only way to utilise and manage the bulk of hair waste recovered from the tannery. It is believed treatment by composting will have future economic benefits to the tanners as well as providing a disposal outlet that is considered environmental-friendly (White *et al.*, 1991; Money, 1991; Money, 1996).

1.4 Composting as a waste treatment method

Composting has a traditional history of being practiced as a waste management strategy. It is regarded as an environmental remediation technology, and widely accepted as an environmentally sound alternative treatment of recycling organic solid waste. This is primarily driven by regulatory pressures to help reduce the amount of solid waste disposed to landfill (Elorrieta *et al.*, 2002; Insam and De Bertoldi, 2007; Pagans *et al.*, 2006; Stentiford, 1987). In

Europe, for example, waste treatment through composting particularly helps member states in meeting the requirements of the waste Landfill Directive (1999). The Directive seeks to reduce as much as possible the amount of biodegradable waste sent to landfill, and has set target levels to be met by all member states. The primary objective of diverting biodegradable organic wastes from the landfill disposal is to reduce the generation of landfill gases, particularly carbon dioxide and methane, which are both green house gases, and groundwater contamination (Directive 99/31/EC; Directive 75/442/EEC).

1.4.1 The Composting process

1.4.1.1 Principles of composting

The composting process may be defined as the biological decomposition of biodegradable organic waste into simpler compounds under controlled aerobic conditions producing a stabilised and sanitised product that is safe to use for agricultural purposes (Adani *et al.*, 1997; Cardenas, 1980; Insam and De Bertoldi., 2007; Vallini, 2002). Composting is a microbial-mediated process which results in the generation of heat, carbon dioxide gas, and water. The general representation of the decomposition of organic matter during composting process is shown in Figure 1.11 (Diaz and Savage, 2007; Fogarty and Tuovinen, 1991; Golueke, 1972; Haug, 1980).

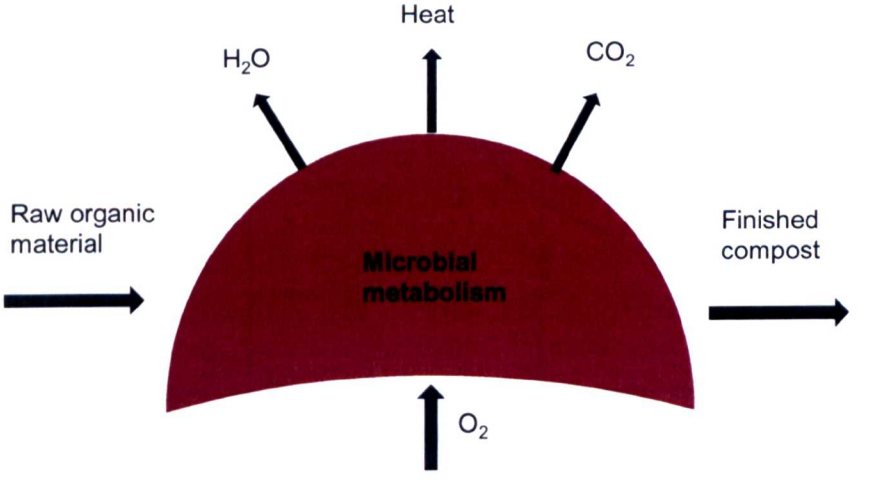

Figure 1.11: The diagram illustrating the composting process for raw organic material, and inputs and outputs of the composting process (Fogarty and Tuovinen, 1991).

Successful decomposition of the organic substrate is the result of cooperative effect of the activity of individual micro-organisms at optimal environmental and nutritional conditions. The composition and diversity of micro-organisms involved (e.g. bacteria, actinomycetes, yeasts, mould, and fungi) changes as composting progresses (Golueke, 1972; Insam and De Bertoldi, 2007; Finstein and Morris, 1975; Strom, 1985). The principal environmental conditions are dependent on temperature, moisture content, oxygen, and pH. Generally, moisture content in the range of 55 to 65% RH and pH between 6.5 and 8.0 is widely accepted as within optimum range for microbial growth and metabolism. Micro-organisms require macronutrients such as carbon, nitrogen, phosphorous and potassium, and certain micronutrients for their growth (Diaz and Savage, 2007; Haug, 1980, Stentiford, 1996; Tiquia *et al.*, 1998; Vallini *et al.*, 2002). Among the macronutrients required, carbon and nitrogen contents are the most influential that affect substrate decomposition throughout composting (Diaz and Savage, 2007; Vallini *et al.*, 2002). The optimum initial proportion of carbon to nitrogen has been reported to be in the range of 20:1 to 40:1, but may vary depending on the substrate (Diaz and Savage, 2007; Haug, 1980; Rynk, 1992).

Temperature is the principal parameter that controls microbial activity during composting (Stentiford, 1996; Vallini *et al.*, 2002). The composting process undergoes four major phases, which are dictated by temperature (Figure 1.12).

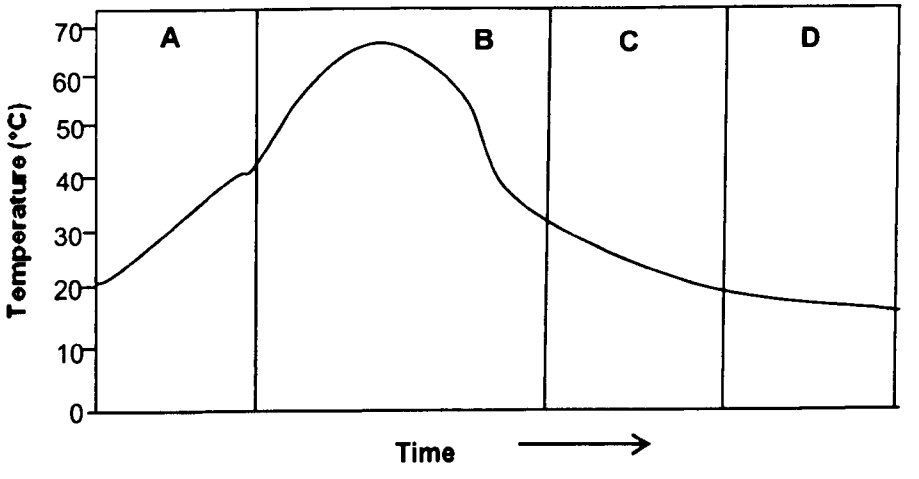

Figure 1.12: Temperature-dependent phases of composting: (A) mesophilic phase; (B) thermophilic phase; (C) cooling phase; (D) maturation phase, (Fogarty and Tuovinen, 1991).

(1) The mesophilic phase: the initial phase of the composting during which the temperature rises from ambient to 40°C due to microbial metabolic activities. The soluble and easily degradable compounds such as monosaccharides, starch and lipids are utilised by the micro-organisms at this initial phase, (2) the thermophilic phase, which is characterised by a high temperature in the range of 40 – 65°C. Thermophilic micro-organisms begin to degrade proteins and other more resistant compounds such as cellulose, hemicelluloses, and lignin. It is believed that most of the microbial decomposition occurs during this phase. Pathogens present are also destroyed during this phase, (3) cooling phase, is characterised by a decrease in temperature (second mesophilic phase) as the microbial population declines due to depletion of readily available organic substrates, and (4) maturation phase is marked by stabilisation and humification of the organic matter (Bernal *et al.*, 2009, 1996; Forgarty and Tuovinen, 1991; Insam and De Bertoldi, 2007). The final product of composting is a stabilised and sanitised organic matter with humic characteristics (Bernal *et al.*, 2009; Garcia, 1991; Senesi, 1989). Humus is the product of the humification process during which compounds of natural origin are transformed into inert humic substances (Aiken *et al.*, 1985).

1.4.1.2 Composting systems

The composting systems available can broadly be assigned to two main categories (Diaz *et al.*, 2007; Haug, 1980; Rynk, 1992; Vallini *et al.*, 2002): (1) open systems, where the organic materials being composted, although may be treated within a building, are not retained in containers, and (2) in-vessel systems, where the organic materials being composted are confined in units referred to as bioreactors. The open systems include (i) windrow composting, and (ii) aerated static pile composting. In-vessel systems refer to composting performed in (iii) vertical reactors, (iv) horizontal reactors (e.g. rotating drums, containers and tunnels). In-vessel bioreactors can also be classified depending on the movement of the material, thus can be denoted as (v) static, and (vi) aerated dynamic trenches, or agitated beds. The type of system to be adopted in any given circumstance generally depends on the availability of land, investment costs, the characteristics and the amount of waste being treated, and regulatory requirements (Diaz *et al.*, 2007; Vallini *et al.*, 2002). In-vessel systems are, however, often considered to be more reliable for greater process control than open systems (Vallini *et al.*, 2002). On regulatory measures, the EU legislative requirements for the disposal of animal by-products not intended for human consumption, enforced by the Animal By-Products Regulation (ABPR, 1774/2002),

recommends in-vessel composting as the appropriate alternative disposal treatment for such animal by-products.

1.4.2 The benefits of compost

Besides reducing the landfill associated problems, the benefits of compost are reported to include a decrease in the volume of waste, improvement of soil structure, water retention and cation exchange capacity, enrichment of organic nitrogen in the soil, and the reduction in potential plant pathogens (Amlinger *et al.*, 2003; Bernal, *et al.*, 2009; Hoitink *et al.*, 1997; Sesay *et al.*, 1997; Tsadilas *et al.*, 2005; WRAP, 2008).

1.4.3 Utilisation of tannery hair waste as a substrate for composting

Although composting has been suggested as the most attractive treatment method for tannery hair waste, there is little information available on the practicalities of utilising hair as a substrate for composting. That is particularly, with regard to processing parameters and knowledge of structural modification of hair during microbial decomposition. In addition, one of the key technological barriers to efficient bioconversion of hair is its resistance to biodegradation due to the complex and highly crosslinked nature of the structure. Since composting relies upon an indigenous community of micro-organisms (Diaz, *et al.*, 2007; Finstein and Morris, 1975) the major challenges, therefore, are to find a biological agent capable of degrading hair, understand their mode of degradation, and favourable process parameters for efficient decomposition of hair in a composting environment.

1.5 Aims and Objectives

The principal aim of this research was to develop an alternative method for the disposal of tannery hair waste through composting. The main research objectives were as follows:

1. To compare the chemical breakdown of black, brown and white bovine hair, specifically when treated with sodium sulfide to evaluate the effect of melanin present in hair.

2. To identify appropriate pretreatment or pretreatments that effectively breaks down the hair structure and enhances its susceptibility to enzymatic digestion. In addition, to understand the characteristic mechanism of hair degradation by the chosen

pretreatment(s) in order to develop a more effective biological treatment as an alternative method of hair treatment.

3. To investigate the biochemical conditions under which the hair structure is completely broken down and how this can be exploited to improve the breakdown of hair during composting. This would be done using the biological alternative strategy developed from the study above (objective number two).

4. To identify a cost-effective source of a biological agent capable of degrading hair with the view of exploiting the potential to enhance hair breakdown during the composting process. To provide a general understanding of the mechanistic breakdown of hair by the identified biological agent, and the optimum conditions of activity.

5. To develop process parameters suitable for composting of hair waste and to analyse the products formed during composting.

CHAPTER 2

ANALYSIS AND PRETREATMENT OF BOVINE HAIR

CHAPTER 2

2. INTRODUCTION

Wool, feathers, and hooves represent naturally resistant waste by-products from animal origin that are produced and processed at an industrial scale into a variety of products, particularly as fertiliser and animal feed ingredients. Similarly, animal-derived proteins such as collagen, gelatine, and casein have been processed into a number of industrial applications (Antunes, 2007, 2008; Catalina *et al.*, 2008; Langmaier *et al.*, 2008; Meena *et al.*, 1999). The industrial-scale processing of these renewable materials is largely driven by increased consumer demands for alternative protein sources, their nutritional value and functional properties (Bourtoom, 2009; De Graaf, 2000). Although animal hair and poultry feathers have a high protein content in the range, 92 – 96% w/w, respectively (Dawber, 1996; Kelly *et al.*, 2006), appropriate processing has to be carried out to improve their nutritional value (Moritz and Latshaw, 2001). A number of methods exist to improve or modify the properties of proteins, broadly categorised into chemical, biological, and physical methods (Bourton, 2009; De Graaf, 2000; Panyam and Kilara, 1996; Shirley and Parsons, 2000). From the aspect of biological (enzymatic) processing of hard-to-degrade animal proteins into other products, pretreatment is considered as a prerequisite. Pretreatment primarily aims to disrupt the natural resistance of the substrate to enhance enzymatic digestibility (Papadopoulos, 1985; Wang and Parsons, 1997).

In the previous chapter, it has been explained that chemical and enzymatic degradation of keratinous materials is limited by their highly covalently crosslinked and complex histological structure, which comprises the cuticle, cortex, and medulla. The stability of each component increases with increasing degree of disulfide crosslinks (Baumann, 1979; Fraser *et al.*, 1972; Gupta and Ramnani, 2006). The cuticle contains a higher proportion of α-keratin (hard keratin), and is known to be responsible for structural rigidity. It forms a physical barrier that surrounds the surface layer of the hair. The cortex, made up of macrofibrils, contains a variable concentration of sulfur proteins, intercellular matrix, melanin granules, and cytoplasmic organelles. The cortex is responsible for integrity but also contributes to the rigidity of the hair. The medullary cells occupying the central part of the fibre contain no keratin (sulfur proteins) and are insoluble (Dawber, 1996; Fraser *et al.*, 1972; Jones, 2001).

Alkali hydrolysis as a pretreatment method to increase the digestibility of keratinous substrates such as feathers has been utilised (Kelly *et al.*, 2006; Papadopoulos, 1985;

Steiner *et al.*, 1983; Wang and Parsons, 1997). Likewise, chemical cleavage of the disulfide bonds (S–S) through oxidation or reduction of cystine have been considered as a prerequisite in studying various aspects of the protein structure, composition, and sequence as well as enhancing proteolytic digestion (Fontana and Gross, 1986; Schrohenloher and Bennett, 1986; Wilkinson, 1986). These treatment methods, however, require significant amount of energy and might not be cost-effective (Tiquia *et al.*, 2005).

2.1 Aims and objectives

The principal aim of this section of research was to identify an effective pretreatment that increased the susceptibility of hair to enzymatic digestion, leading to a complete breakdown of the hair structure. Subsequently, to use the observed mechanism of hair degradation to develop an efficient natural biological system that can achieve a similar effect. In the previous chapter it was explained that there has been unconfirmed belief in the tanning industry that during the conventional hair dissolving process, using sodium sulfide and lime, black and brown hair are solubilised more slowly than white hair. Therefore, in addition to the principal aim, the key objectives of this chapter were:

1. To carry out physicochemical analysis and characterisation of bovine hair, using microscopy, Differential Scanning Calorimetry, Total Kjeldahl Nitrogen and electrophoresis methods.
2. To compare the effect of solubilisation of melanised brown and black bovine hair with white bovine hair following chemical treatment with sodium sulfide,
3. To carry out physical and chemical pretreatment of bovine hair, by heating, alkali hydrolysis and use of reducing agent, to devise an effective method for degrading hair.
4. To analyse the products formed and the extent of structural modifications of hair due to such treatments.

2.2 Materials and Methods

Unless otherwise specified, the commercial reagents used in the experiments were of analytical grade obtained from Sigma Aldrich, UK. All standard acid solutions were provided by Fisher Scientific, UK. All the analyses were carried out in duplicate.

2.2.1 Sampling and sample preparation

Raw materials: hair samples from black, brown, and white bovine hides from the British School of Leather Technology (BSLT, University of Northampton) were obtained using an electric clipper, washed with deionised water and dried at ambient conditions. The dry samples were placed in polyethylene resealable bags, labelled and stored until required (Figure 2.1). Unless otherwise stated, before use the samples were solvent extracted to remove surface lipids and grease by Soxhlet extraction based on the AOAC Official Methods of Analysis (1990). Before the experiments were carried out references assigned to hair samples were as follows: brown hair (Brown), black hair (Black), and white hair (White).

Figure 2.1: Degreased intact bovine hair samples: A =White, B = Black, and C = Brown.

2.2.2 Physicochemical analysis of intact hair

2.2.2.1 Physical structural analysis

The objective was to examine the physical structure of intact bovine hair, as a starting material, using scanning electron microscope (SEM) in order to reveal and compare the various histological components namely, the cuticle, cortex, and medulla. It was important to observe and develop an understanding of the physical conditions of these components in their intact and undegraded state.

2.2.2.1.1 Sample preparation and scanning electron microscopy (SEM)

Hair samples were washed with phosphate buffer, pH 7.5, fixed in 1% (v/v) glutaraldehyde and stained with 0.1% (v/v) osmium tetroxide according to the procedure by Wagner and Bailey (1999). Stained samples were dehydrated in 70% (v/v) and 96% (v/v) alcohol and mounted on aluminium stubs using an adhesive carbon mount. The mounted samples were coated with gold in vacuum and examined using scanning electron microscope (Hitachi S-3000N, Japan).

2.2.2.2 Determination of nitrogen

The percentage nitrogen in hair was determined by the Total Kjeldahl Nitrogen (TKN) method as described by Vogel (1961). All the determinations were carried out in duplicate. Full description of the procedure is provided in Appendix A.

2.2.2.3 Ash content

The ash content determination was performed by the wet-ashing procedure as described by Allen (1989). Full description of the procedure is provided in Appendix B.

2.2.2.4 Extractable lipids

Grease and surface lipids were extracted with dichloromethane (Fisher Scientific, UK), for 4 hours by Soxhlet extraction according to The Association of Official Analytical Chemists Official Methods of Analysis (AOAC, 1990). Full description of the procedure is provided in Appendix C.

2.2.2.5 *Thermal analysis using Differential Scanning Calorimetry (DSC)*

Differential scanning calorimeters (DSC) have been widely used to determine the thermodynamics of phase transitions and conformational changes in biological systems such as proteins. Measurements have included the determination of stability of proteins (Hinz and Schwarz, 2001; Bischof and He, 2005; Ehen *et al.*, 2004). The major parameters measured by the DSC are the glass transition, denaturation temperatures, and enthalpic changes (ΔH) associated with such thermal events. Enthalpy (ΔH) is the energy absorbed by the sample, and a quantitative result depends on the determination of the area generated under the peak curve with reference to a baseline (Bruylants *et al.*, 2005).

2.2.2.5.1 Sample preparation

Black, brown and white hair samples for analysis with (DSC) were cut and ground using a pestle and mortar then passed through a 36 μm sieve to obtain uniform particle size. The samples were conditioned at 21 ± 1°C and 60% RH (relative humidity) for 48 hours.

2.2.2.5.2 Measurement by Differential Scanning Calorimetry (DSC)

The sample (9 mg) was weighed into a 40 μl medium pressure aluminium crucible and tightly sealed. Thermal analysis was performed using the DSC (Mettler Toledo DSC 822, Japan) using an aluminium pan as a reference. The temperature was programmed from 20°C to 250°C, at a heating rate of 5°C min^{-1}. Each sample was run in duplicate. The results obtained from thermal analysis of intact hair (non-treated) samples were used as reference controls.

2.2.2.6 *Characterisation of hair proteins using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).*

In order to understand the differences between brown, black and white bovine hair it is necessary to compare the basic structure of their constituent proteins. A method that is commonly used for protein analysis, for purity and molecular weight estimation is electrophoresis, which makes use of a strong protein-dissociating detergent sodium dodecyl sulfate (SDS). The electrophoretic analysis of proteins and peptides is often carried out in gels of crosslinked polyacrylamide (PAGE), which acts as a molecular 'sieve', for the proteins migrating through the gel matrix and are separated proportionately according to their charge-to-mass ratio. Hence, the technique is referred to as Sodium Dodecyl Sulfate

Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Andrews, 1986; Hames and Rickwood, 1990; Walker, 2002; Weber and Osborn, 1969). In the case of hair SDS electrophoresis is carried out on extracted and modified proteins. The modification is achieved by alkylation of the reduced proteins with iodoacetamide producing stable S-Carbamoylmethylated (Scam) keratin derivatives (Fraser *et al.*, 1972; Katsuumi *et al.*, 1989).

2.2.2.6.1 Extraction of hair keratin proteins

To produce soluble proteins for analysis with SDS-PAGE hair samples, 5 mg, were extracted under nitrogen with 0.5 ml of 0.05 M Tris-HCl buffer, pH 9.3, containing 8 M urea (BDH, UK) and 0.05 M dithiothreitol (DTT) (Fisher Scientific, UK) for 18 hours at room temperature, according to Marshall, (1983). The extraction was carried out under constant agitation at 100 revolutions per minute (r.p.m) in an orbital incubator (S1 50 Stuart Scientific, UK). The extractions were performed in duplicate.

2.2.2.6.2 Modification of reduced keratins

Modification of reduced hair proteins was according to the procedure of Aitken and Learmont (2002). Briefly, the reduced hair proteins were alkylated using 50 μl of 0.5 M iodoacetamide in 2 M Tris-HCl buffer, pH 8.0, at 37°C in darkness with constant agitation at 100 r.p.m in an orbital incubator (S1 50 Stuart Scientific, UK) for 30 minutes. This converted the reduced proteins to *S-Carbamoylmethylated* (Scam) derivatives. The samples were cooled to room temperature and excess iodoacetamide reacted with 2-mercaptoethanol (Fisher Scientific, UK) for 10 minutes. The solution was centrifuged (Jouan GR20.22) at 15,000 r.p.m, and 4°C for 15 minutes. The recovered protein was dialysed at 4°C overnight against 0.05 M ammonium bicarbonate buffer, pH 7.9, and then centrifuged at 5000 r.p.m and 4°C for 20 minutes to remove any impurities.

2.2.2.6.3 Protein determination

The concentration of protein in the solubilised hair keratin was determined based on the method of Lowry *et al.*, (1951) and described by Waterborg (2002). The determination was carried out in duplicate, with Bovine serum albumin (BSA) as protein standard. The absorbance was measured at 500 nm on a UV 2501PC spectrophotometer (Shimadzu, Japan). Full description of the protocol is provided in Appendix D. The determination of total protein concentration in hair was carried out by the method of total hydrolysis of protein by acid (6 M HCl) for 18 hours at 110°C as described by Darbre (1986).

2.2.2.6.4 Electrophoresis of the modified (Scam) keratin derivatives

The SDS-PAGE of the *S-Carbamoylmethylated* (Scam) keratin derivatives was carried out according to the method of Laemmli (1970). The proteins were resolved in 15% (v/v) polyacrylamide gel with a 4% (v/v) stacking gel under a steady current of 2 Amp at 75°C on a Bio-Rad (UK) power supply apparatus (Bio-Rad model 200/2.0) until the dyeing track of the sample buffer reached the bottom edge of the gel. Proteins in the gel were stained overnight with 0.2% (w/v) Coomassie brilliant blue G-250 and destained with 10% (v/v) methanol and 7% (v/v) acetic acid until a clear background was obtained. The molecular weight of the protein samples were estimated against a Sigma low-molecular weight protein standard marker range 6 – 66kD. The composition and preparation of the gels are presented in Appendix E. The preliminary optimisation studies (modification of keratin and gel concentration) are presented in Appendix E1 (Figures E1 – E3).

2.2.3 Physical treatments

2.2.3.1 Heat treatment

Heating is one of the most common denaturing methods used for proteins, since the stability of proteins is limited at high temperatures (Jaenicke, 1990; Jaenicke and Bohm, 1998; Putman, 1953). This is achieved by increased kinetic energy which causes the disruption of the intra-molecular hydrogen, hydrophobic bonds, as well as van der Waals forces (Jaenicke, 1991, 2000). The advantage of heat denaturation is that it is easy to apply (Leman and Dolgan, 2004).

Bovine hair samples (black, brown and white) (0.1 g) were each, separately, placed in duplicate into a 250 ml beaker containing 100 ml of deionised water. The samples were heated from 30°C to 100°C on a digital hot plate fitted with a temperature probe sensor (SD162 Stuart, UK). Once the required temperature was reached the heating was allowed to progress for 10 minutes longer. This was considered to be of a sufficient period to allow heat distribution within the sample, and therefore, allowing denaturation. The samples were filtered using Whatman No. 1 filter paper and dried at ambient conditions. The dry samples were prepared for thermal analysis according to Section 2.2.2.5.1. Thermal analysis was performed using DSC as described in Section 2.2.2.5.2.

2.2.4 Chemical treatments

The chemical pretreatment methods were selected on the basis of the following factors:

- Alkali is capable of breaking the hydrogen bonds and salt-linkages present on the polypeptide chain of protein. The alkali used was sodium hydroxide (NaOH).
- Reducing agent specifically cleaves the disulfide crosslinks (S–S) present in keratin. The reducing agent used was potassium thioglycolate (KTG).
- Urea and guanidine hydrochloride were used as hydrogen bond breakers: the reagents denatures the protein by acting on the non-covalent bonds present in protein (Alonso and Dill, 1991; Shellman, 1987; Wetlaufer *et al.*, 1964).

The study was divided into two sections: (1) the treatments of bovine hair with sodium sulfide to compare the rate at which melanised bovine hair (brown and black) and white hair were solubilised. (2) Chemical pretreatments: for the purpose of elucidating the breakdown mechanism and enhancing enzyme digestibility of bovine hair. The degree of chemical and physical modification was also compared between the samples. For each chemical pretreatment, the reaction was carried out for one hour which was considered enough time to cause chemical and physical structure changes in hair. All the experiments were carried out in duplicate.

2.2.4.1 Treatment of hair with sodium sulfide

To determine the effect of sodium sulfide on black, brown, and white hair, two experiments were performed. One study was carried out in the tannery under drum conditions to simulate the conventional unhairing process and the amount of hair keratin solubilised measured as Total Kjeldahl Nitrogen (TKN) (Section 2.2.2.2). The second experiment was carried out in the laboratory to compare the effect of sodium sulfide treatment on the thermal stability of brown, black and white bovine hair.

2.2.4.1.1 Tannery studies using sodium sulfide

Black, brown, and white bovine hair samples (2 g) were respectively placed in 2 litre demonstration drums, and a solution (150 ml) containing 0.07 M of tannery grade sodium sulfide (BSLT, UK) added. The concentration of sulfide used was previously optimised to gradually solubilise the hair, thus allowing the solubilisation process to be monitored. Each

experiment was carried out in duplicate. The drums were operated for 0.5, 1, 2, 3, and 5 hours at 20°C. A blank control containing only hair and deionised water was run at the same time. After each period the samples were removed, the solution recovered and filtered through Whatman filter paper. The filtrate (50 ml) from each experiment was used for the determination of Total Nitrogen (Section 2.2.2.2).

2.2.4.1.2 Laboratory studies using sodium sulfide

For each bovine hair type (black, brown and white), sample (0.01 g) was placed into duplicate 50 ml quick-fit flasks, and a 2 ml of 0.07 M sodium sulfide solution was added. The flasks were flushed with nitrogen to prevent re-oxidation of the disulfide bonds and then tightly sealed. The reduction was carried out at 20°C in an orbital incubator agitating at 100 revolutions per minute for 1, 2, 3, and 5 hours. To ensure the stability of the reduced keratins the samples were further modified by reacting with 100 µl of 0.5 M iodoacetamide in 2 M Tris-HCl buffer pH 8.0 at 37°C. The reaction was carried out in darkness with constant agitation at 100 revolutions per minute in an orbital incubator for 30 minutes. The samples were cooled to room temperature. Hair samples were recovered by filtering the solution with Whatman No.1 filter paper and then rinsed with deionised water. The dry samples were prepared and subjected to thermal analysis with Differential Scanning Calorimetry (DSC) as described in Sections 2.2.2.5.1 and 2.2.2.5.2, respectively.

2.2.4.2 Alkaline (sodium hydroxide) pretreatment (NaOH)

Samples (0.05 g) of black, brown and white bovine hair were respectively placed into duplicate 50 ml quick-fit flasks, and a 3 ml solution of 0.1 M sodium hydroxide (pH 12) was added. Each flask was flushed with nitrogen and sealed. The hydrolysis was carried out at 40°C in an orbital incubator agitating at 100 r.p.m for 60 minutes. Control samples contained each sample type of hair and deionised water, without sodium hydroxide. After hydrolysis, the samples were incubated with 0.5 M iodoacetamide (300 µl) in 2 M Tris-HCl buffer, pH 8.0, at 37°C in darkness with constant agitation at 100 r.p.m in an orbital incubator for 30 minutes then cooled to room temperature. Hair samples were recovered by filtration using Whatman No.1 filter paper, washed with deionised water, and dried at ambient conditions. The dry hair samples were prepared and subjected to thermal analysis using DSC (Section 2.2.2.5.2). Structural modification of hair was analysed using scanning electron microscope (SEM) (Hitachi S-3000N, Japan) as described in section 2.2.2.1.1. The filtrate was dialysed in deionised water for 24 hours at 4°C and then centrifuged (Jouan GR20.22) at 5000 r.p.m

and 4°C for 20 minutes. The protein concentration in the filtrate was estimated by the method of Lowry *et al.*, (1951) (Section 2.2.2.6.3).

2.2.4.3 Pretreatment with potassium thioglycolate (KTG)

The objective of this experiment was to evaluate the physicochemical transformation of hair following a pretreatment with a reducing agent targeting the disulfide bonds. The experimental procedure was as described in Section 2.2.4.2. Briefly, each sample of bovine hair (black, brown and white) (0.05 g) was treated with 3 ml of 0.1 M potassium thioglycolate (Fisher Scientific, UK) in 0.2 M Tris-HCl buffer, pH 10, in the absence of oxygen at 40°C in an orbital incubator, agitating at 100 r.p.m for 60 minutes. Control samples contained hair and the buffer without the reducing agent. The samples were recovered and prepared for thermal analysis (DSC) as described in Sections 2.2.2.5.1 and 2.2.2.5.2; microscopy (SEM) Section 2.2.2.1.1; and protein analysis (Section 2.2.2.6.3).

2.2.4.4 Pretreatment with sodium hydroxide plus potassium thioglycolate (NaOHKTG)

The objective of the experiment was to evaluate the effects of solubilisation and structural modification of hair using a combined treatment of a reducing agent and an alkali. The experimental procedure was as described in Section 2.2.4.2. A brief outline is as follows:

Each of the bovine hair samples (black, brown and white) (0.05 g), was placed in duplicate into 50 ml quick-fit flasks and a 3 ml solution of pH 10, containing 0.1 M potassium thioglycolate and 0.1 M sodium hydroxide, was added. The flasks were flushed with nitrogen and incubated at 40°C under agitation for 60 minutes as described in Section 2.2.4.2. Blank controls contained hair with Tris-HCl buffer (pH 10) without potassium thioglycolate and sodium hydroxide. The samples were recovered and prepared for thermal analysis (DSC), Sections 2.2.2.5.1 and 2.2.2.5.2; microscopy (SEM), Section 2.2.2.1.1; and protein analysis (Section 2.2.2.6.3).

2.2.4.5 Pretreatment with urea and guanidine hydrochloride

Bovine hair (black, brown, and white), 0.05 g, were separately placed in duplicate into 50 ml quick-fit flasks and 3 ml solution containing either 8 M urea (BDH, UK) in 0.2 M Tris-HCl buffer pH 10, or 6 M guanidine (Sigma Aldrich, UK) in 0.2 M Tris-HCl buffer pH 10, was added. Blank controls contained hair and Tris-HCl buffer pH 10 without urea or guanidine. All other experimental procedures were as described in section 2.3.3.2. The samples were

recovered and prepared for thermal analysis (DSC), Sections 2.3.2.5.1 and 2.3.2.5.2, microscopy (SEM), Section 2.3.2.1.1, and protein analysis (Section 2.2.2.6.3).

2.2.5 Assessment of structural damage

The effectiveness of each particular pretreatment was evaluated based on the following parameters:

- The physicochemical changes in terms of: (i) the concentration of solubilised protein, and (ii) the extent of physical structural damage, assessment by microscopic examination (SEM).

- Effect on thermal stability as measured by DSC.

2.3 Statistical analysis

The results of the effects of different treatments (thermal stability and protein solubilised) were analysed using SPSS 17.0 statistical software package. The results are given as mean values (*i.e.* mean ± confidence interval), and were compared using one-way analysis of variance (ANOVA) with a test significance level of $p \leq 0.05$, and 95% confidence interval.

2.4 Results and discussion

2.4.1 Physicochemical analysis

2.4.1.1 Physical structure

Figures 2.2 and 2.3 represent the microscopy (SEM) micrographs of the morphology of the major histological components of intact bovine hair as described in Chapter 1, Section 1.3.2.3. On the outer surface of the fibre is the cuticle (A) characterised by the scaly sheet-like structures (Figure 2.2). The cortex (B) makes up the bulk of the hair and in the central part of the hair is the medulla (C), composed of vacuolated air-filled cells (Figure 2.3).

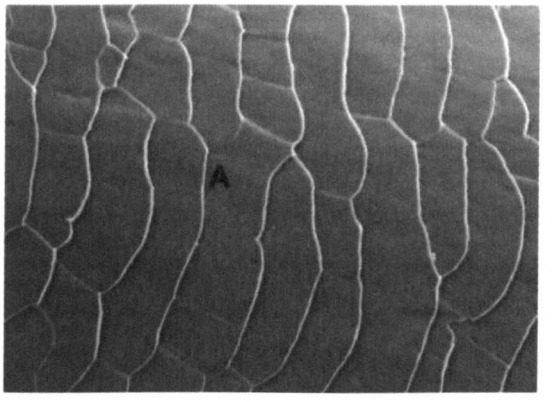

Figure 2.2: SEM image of the surface of intact hair shaft: (A) cuticle (mag. x1.0K, 5.0kV).

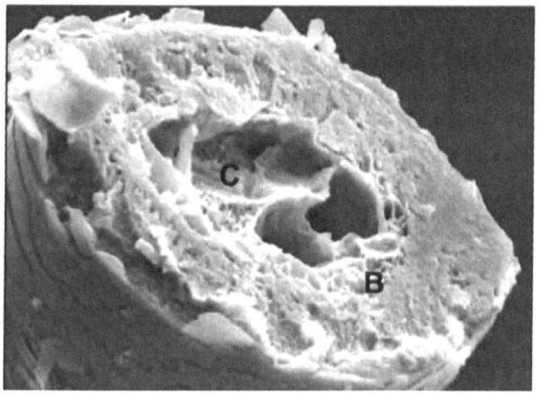

Figure 2.3: SEM image of the cross-section of an intact hair shaft: (B) cortex, and (C) medulla (mag. x1.0K, 5.0kV).

From the microscopy (SEM) examination there were no histological differences observed between the samples (black, brown and white bovine hair).

2.4.1.2 Thermal analysis (Differential Scanning Calorimetry)

Figure 2.4 shows representative thermal curves for the transition temperatures of intact black, brown and white bovine hair samples. The samples produced two major endothermic peaks, hereby designated as T_g for glass transition (Peak 1), which is attributed to the glass transition temperature of the α-helical structures, and T_D for denaturation temperature (Peak 2), which is associated with the denaturation or melting of the α- helical structures of α-keratins (Cao, 1999; Cao and Leroy, 2005; Milczarek *et al.*, 1992; Phillips, 1985; Wortmann *et al.*, 2006). The observed shape of the thermal calorimetric curves is characteristic to the double peaks observed in keratin tissues such as wool, human hair, and feathers (Manich *et al.*, 2005; Tonin *et al.*, 2004).

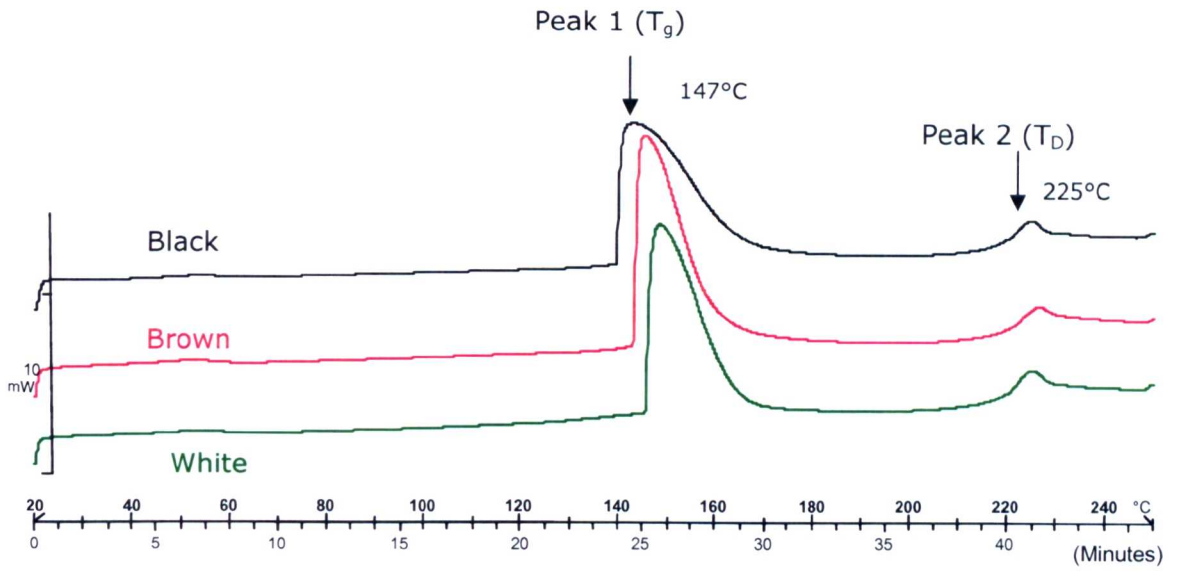

Figure 2.4: Thermal Temperature transition curves of intact black, brown and white bovine hair (control samples) (mean temperature shown on the curve). Samples were heated from 20 – 250°C at 5°C min^{-1}.

Table 2.1 is a summary of the average transition temperatures obtained from the thermal plots measured by the DSC. The area under each of the peak curve yields the enthalpy (ΔH), which is the energy required for the transition and denaturation of the α-helices. For bovine hair keratins the average thermal glass transition peak (T_g) obtained between the different hair samples was 147 ± 1.8°C, with an enthalpy (ΔH_g) of 149 ± 3 J/g. The thermal denaturation temperature (T_D) was 225 ± 1.9°C, with an enthalpy (ΔH_D) of 5.2 ± 1.4 J/g.

Table 2.1: Thermal transition temperatures of intact bovine hair samples as measured by DSC from 20 – 250°C at 5°C min^{-1}.

	Peak 1		Peak 2	
Hair sample	**Transition temp. (°C)***		**Transition temp. (°C)***	
	T_g	ΔH_g (J/g)	T_D	ΔH_D (J/g)
Brown	147 ± 1	149 ± 6	227 ± 2	4.7 ± 2.2
Black	147 ± 1	151 ± 1	224 ± 1	5.5 ± 1.7
White	146 ± 2	148 ± 4	225 ± 1	5.3 ± 1.1

*Mean of six duplicates ± confidence interval

The results of the thermal transition temperatures were consistent with the range of values obtained from other α-keratin fibres such as wool and human hair. Milczarek *et al.* (1992), observed a glass transition temperature at 155°C and a thermal denaturation peak at 233°C in human hair keratins. On the other hand, Wortmann *et al.* (2006) observed that the glass transition temperature of human hair is in the range of 135 – 144°C. While studying thermal

properties of merino wool, Manich *et al.* (2005) observed the double endotherms at 117.9°C and enthalpy (ΔH) of 164.36 J/g on the first peak, and 227.4°C and enthalpy of 3.89 J/g on the second peak.

In this study, the results of mean values of temperature of glass transition (147 ± 1°C) and enthalpy of 149 ± 3 J/g, denaturation temperature (227 ± 2°C) and enthalpy of 5.2 ± 1.4 J/g were used as reference values for the intact bovine hair (control sample). A comparison between the profiles of thermal calorimetry curves of different hair types (black, brown and white hair) analysed showed that although there were slight variations there was a similarity between the samples, and that the glass transition temperature (T_g) was similar, and the denaturation or melting (T_D) of the crystalline α-helices occurred within the same temperature range. This may suggest that the presence of melanin in hair does not alter its basic thermal properties. A physical examination of the samples at the respective peaks showed that the samples experienced thermal degradation by the end of the DSC run at 230°C. The samples were carbonised at temperatures above 225°C (Figure F1, Appendix F), which further suggests that the melting temperature to be within the range of 225°C to 230°C.

2.4.1.3 Chemical analysis

Table 2.2 shows the results of the mean values of the total nitrogen, ash content and extractable lipids analysis carried out on brown, black and white bovine hair samples. The mean percentage value of the total nitrogen content of bovine hair as measured by the TKN method (Section 2.2.2.2) was 15.3 ± 0.5% (dry weight). A comparison of the mean values of total nitrogen of the intact control hair samples (black, brown and white) analysed showed there was no significant difference between the samples ($p > 0.05$). However, the ash content varied between the samples, with the mean percentage value obtained from black hair (3.6 ± 0.1% w/w) showing significant difference ($p < 0.05$) from the others.

Table 2.2: Total Nitrogen, ash content and extractable lipids of brown, black and white bovine hair samples, (percentages based on dry weight of sample).

	Brown hair (%)*	**Black hair (%)***	**White hair (%)***
Nitrogen	15.0 ± 0.4	15.0 ± 0.4	15.8 ± 0.4
Ash content	1.6 ± 0.4	3.6 ± 0.1	1.2 ± 0.1
Extractable lipids	0.3 ± 0.2	0.5 ± 0.1	0.5 ± 0.1

*Mean of six duplicates ± confidence interval

As was explained in Chapter 1 (Sections 1.3.2.2 & 1.3.2.5) trace elements can be incorporated in hair from different sources, both exogenous and endogenous, and that among the biological functions of melanin is its ability to chelate heavy metals (Cubarsi *et al.*, 2006; Dawber, 1996; Riley, 1997). Therefore, it is likely that the variability in the ash content may be attributed to the incorporated elements arising from (1) environmental and nutritional sources (Mathew, 1954), and possibly (2) melanin content. Thus it is likely melanised hair may exhibit high ash content as result of its metals chelation properties (Meredith and Sarna, 2006; Riley, 1997). The mean percentage value of extractable lipids was 0.43 ± 0.1%. A comparison between the intact hair samples (black, brown and white) analysed showed no significant difference.

2.4.1.4 Electrophoresis of brown, black and white bovine hair using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis technique has been used successfully to characterise keratin proteins, and in the identification of species as well as taxonomic studies (Carracedo *et al.*, 1987; Gillespie and Inglis, 1965; Marshall, *et al.*, 1977). Figure 2.5 shows the electrophoretic characteristics of *S-carbamoylmethyl* (SCam) keratins extracted from the three different types of bovine hair samples, *i.e.* black, brown and white hair.

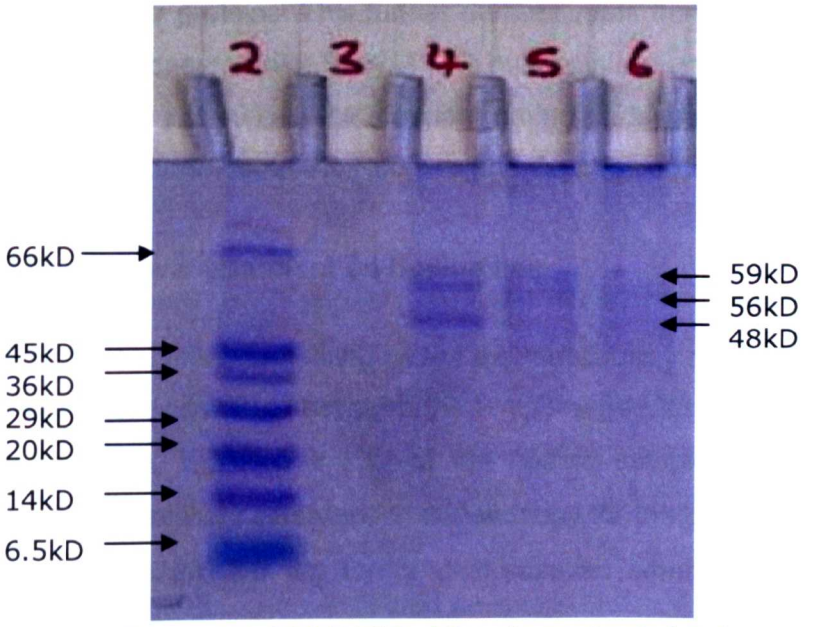

Figure 2.5: SDS-PAGE of *S-carbamoylmethylated* bovine hair keratins (SCam). Lane (2): standard, (3): buffer, (4): white hair, (5): black hair, and (6): brown hair

The electrophoresis shows the distribution pattern of the extracted hair proteins stained with Coomassie brilliant blue G-250. Lane 2 shows the Sigma low molecular weight protein

standard, lane 3 is the dilution buffer, while lanes 4, 5, & 6 represents the electrophoretic mobility of white, black and brown bovine hair respectively.

Protein structural information is important in the purification and separation of protein mixtures, as well as providing useful information when comparing different proteins, which otherwise might not be detected by native sequence information alone (Le *et al.*, 2009; Marshall and Inglis, 1986). Although differences may exist in the distribution of the molecular weights of proteins within species and inter-species (Gillespie, 1968; Woods and Orwin, 1987), however, in this study, the electrophoretic distribution of the extracted bovine hair proteins were resolved at three major molecular weight band ranges of 48kDa – 59kDa (Figure 2.5). The samples exhibited similar electrophoretic mobility and molecular weight distribution. The similarity in the molecular weight distributions as evidenced by the electrophoretic patterns demonstrates that all the samples (black, brown, and white bovine hair) have similar protein constituents and molecular architectural characteristics. In addition, this similarity in the molecular weight resolution and distribution suggests that melanin may not form part of the architectural molecular structure of hair.

Although melanin granules are tightly held within the keratin matrix of the hair cortex (Liu *et al.*, 2003) their structural role would appear to be mechanical rather than as part of the molecular structure of hair proteins. This further demonstrates that in spite of their ability to crosslink with proteins (Bonser, 1995; Riley, 1997) melanins are not proteins since they lack the peptide bonds and do not contain the standard amino acids which are the basic building blocks of proteins (Liu and Simon, 2003; Rodgers and Dean, 2000).

2.4.2 Effect of heat treatment on bovine hair

Table 2.3 summarises the effect of heating on the thermal stability of bovine hair keratin. The data shows no significant difference between the temperatures for glass transition, T_g, (144 ± 1°C) and denaturation, T_D, (225 ± 1°C) of the heated samples and control samples. However, there was a significant difference in the enthalpy for the glass transition (ΔH_g), 122 J/g, and denaturation (ΔH_D), 2.5 J/g for heated samples when compared with control samples.

Table 2.3: Average thermal transition temperatures observed from the thermal plots measured by DSC from 20 – 250°C at 5°C min^{-1}, for brown, black and white bovine hair samples previously heated in temperature range 30 - 100°C.

Sample	Peak 1		Peak 2		Protein solubilised (mg/ml)
	Transition temp.(°C)*		Transition temp.(°C)*		
	T_g	ΔH_g (J/g)	T_D	ΔH_D (J/g)	
Brown (control)	147 ± 1.0	149 ± 9.0	227 ± 2.0	4.7 ± 1.2	
Brown (heated)	145 ± 1.5	124 ± 12	226 ± 1.3	2.0 ± 0.4	Undetectable
Black (control)	147 ± 1.0	151 ± 6.0	224 ± 1.0	5.5 ± 1.7	
Black (heated)	144 ± 1.2	123 ± 9.0	225 ± 2.4	2.4 ± 0.5	Undetectable
White (control)	146 ± 2.0	148 ± 4.0	225 ± 1.0	5.3 ± 1.1	
White (heated)	144 ± 1.2	119 ± 13	224 ± 1.0	3.0 ± 0.3	Undetectable

*Mean of six duplicates ± confidence interval

There was a decrease in ΔH_g by 18% from an average of 149 J/g for the intact hair (control) to 122 J/g for heated hair samples. On the other hand the enthalpy for denaturation (ΔH_D) was lowered by 52% from an average of 5.2 J/g for the intact hair (control) to 2.5 J/g for heated hair samples. This decrease indicates heat treatment may have caused fission of hydrogen bonds, which constitute part of the stabilising forces of keratin. The fission of hydrogen bonds is consistent with observations made by Sweetman (1967), who pointed out that heating wool up to 100°C may cause hydrothermal degradation.

In this particular experiment, although thermal properties of hair appeared to have been modified, soluble proteins were not detected in the filtrate. This may suggest that the particular treatment used (*i.e.* heating from 30 – 100°C) did not cause hydrolysis of the polypeptide, or the concentration of soluble protein in the filtrate was less than the range detected by the analytical method used. There was no apparent physical modification of the structure of the hair samples following the heat treatment. The results may be interpreted to mean that heating within the temperature range applied (30 – 100°C) may not be suitable as a single pretreatment option. However, heating can be used to enhance other pretreatment processes.

2.4.3 Chemical Treatments

2.4.3.1 Effect of hair treatment with sodium sulfide (tannery)

Figure 2.6 shows the percentage nitrogen as measured by the TKN method from solutions of sodium sulfide-reduced hair. The nitrogen content of the solution was expressed as a percentage of the total nitrogen content in the dry hair sample. The percentage nitrogen of the dry hair amounted to 15%. The total nitrogen content in 2 g of dry hair sample was calculated to be 0.3 g. The results show that in the initial half hour of the experiment, there was no significant difference between the samples in the percentage nitrogen of hair keratin solubilised. The percentage nitrogen of solubilised keratin detected using TKN during this time from brown hair amounted to 1.7% (w/v), black, 2.8% (w/v), and white hair, 2.6% (w/v). A significant difference was observed to occur after one hour when the percentage nitrogen of soluble keratin detected from white hair increased from 2.6% (w/v) to 7.3% (w/v), whilst brown and black hair showed only marginal increases of 2.2% (w/v) and 3.3% (w/v) of soluble nitrogen respectively. In two hours the percentage of soluble nitrogen detected in white hair increased to 34% (w/v). At the same period the soluble nitrogen detected from brown hair was approximately 4% (w/v) and black hair 7.7% (w/v).

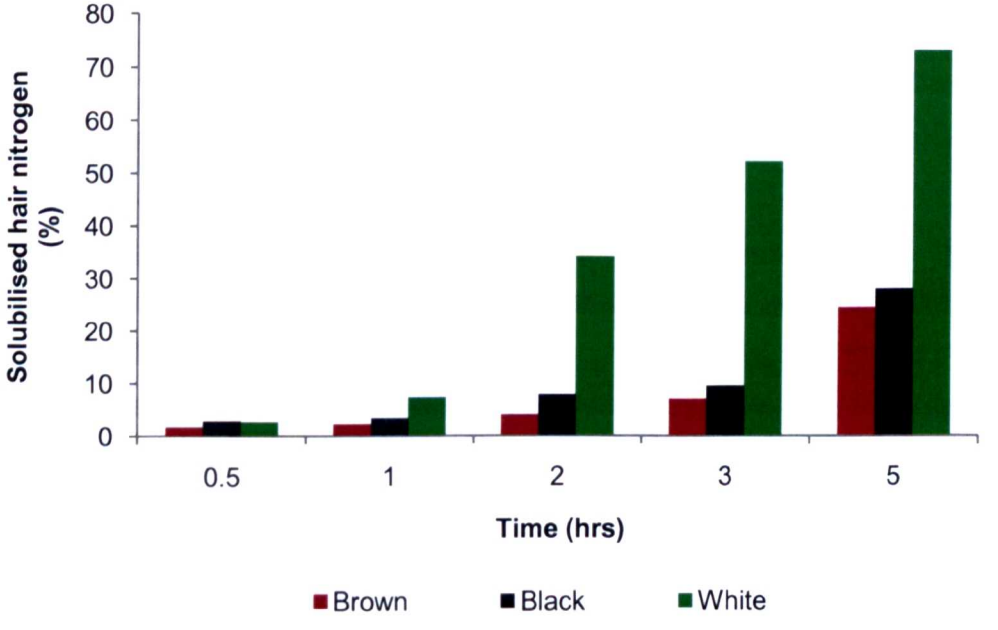

Figure 2.6: The comparative effect of sodium sulfide solubilisation of brown, black and white bovine hair keratin for 0.5 – 5 hours at 20°C in a simulated tannery drum unhairing conditions.

The percentage of solubilise nitrogen from white hair increased to 52% (w/v) after three hours while 7% (w/v) and 9.4% (w/v) of solubilised nitrogen was detected in solutions from brown and black hair samples, respectively. At the end of the experimental period (five

hours) the solubilised protein, measured as total nitrogen, from white hair amounted to 73% of the weight of total nitrogen of the original material. Comparatively the measured nitrogen in solutions from brown and black hair samples at the same period was 24.4% (w/v) and 28% (w/v), respectively. On the basis of the measured percentage of solubilised nitrogen, white hair seems to undergo rapid solubilisation by sodium sulfide than melanised brown and black hair samples.

The combined results from the molecular characteristics (Section 2.4.1.4) and thermal properties of intact bovine hair (control samples) (Section 2.4.1.2) indicate that the samples share similarities in these basic, but fundamental, properties. Importantly, these results demonstrate that the presence of melanin does not change these properties. The stability and resistance of hair to chemical degradation is mainly attributed to the densely crosslinked disulfide bonds. The cortex contains 85 – 90% of hair proteins (dry weight). Melanin exists as an amorphous and a highly negatively charged insoluble biopolymer, which is distributed and tightly held within a keratin matrix of the cortex (Fraser *et al.*, 1972; Liu *et al.*, 2003; Prota, 1980; Riley, 1997). At alkaline conditions of pH 10 and above sodium sulfide ionise. The sulfide anions (S^{2-}) become the predominant reactive species that will participate in the nucleophilic substitution reaction with disulfide linkages of cystine according to the reaction in Scheme 2.1, shown below (Bienkiewicz, 1983; Covington, 2009).

Scheme 2.1: Sulfide anions (S^{2-}) nucleophilic substitution reaction with disulfide crosslinks of cystine (arrow). (R = polypeptide chain)

On the basis of the highlighted issues, it may be suggested that the slow chemical degradation of brown and black bovine hair samples during treatment with sodium sulfide is due to the slow diffusion of S^{2-} ions into the cortical matrix, due to the presence of larger melanin molecules. The larger melanin molecules may have caused limited accessibility of the intra-chain disulfide crosslinks and their availability for chemical interaction. This is further demonstrated by the fact that solubilisation is seen to increase with time. It is also possible that at a high pH, ionisation of the acidic side chains (COOH group) present in the melanin, is likely to create a build up of negative charges on the pigment, which may form an effective electrostatic barrier to the penetration of the sulfide anions (S^{2-}), thus affecting the rate of reduction. This phenomenon (the build up of negative charges on the pigment) has been described as a possible rate-limiting factor during hair bleaching with hydrogen peroxide (Wolfram *et al.*, 1970). In addition, the relative resistance of melanin to degradation

and its insolubility (Casadevall *et al.*, 2000) may have contributed to a slow chemical modification of the pigmented samples.

2.4.3.1.1 Damage assessment using Scanning Electron Microscope

Figures 2.7 – 2.12 are representative microscopy (SEM) images showing the progressive structural modification of brown, black and white bovine hair following treatment with sodium sulfide. It is evident from the micrographs that within the first three hours the white hair experienced extensive structural damage (Figure 2.9) compared to brown and black hair (Figures 2.7 & 2.8).

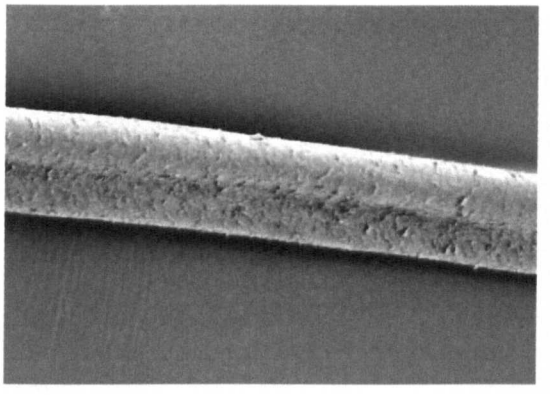

Figure 2.7: SEM image of brown hair illustrating damage to the hair shaft following 3 hours treatment with sodium sulfide (mag. x200, 5.0kV).

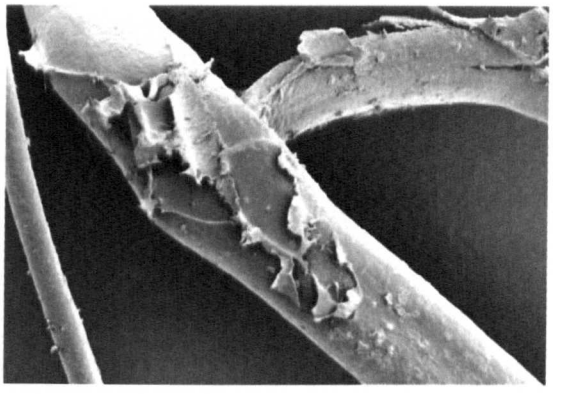

Figure 2.8: SEM image of black hair illustrating damage to the hair shaft following 3 hours treatment with sodium sulfide (mag. x200, 5.0kV).

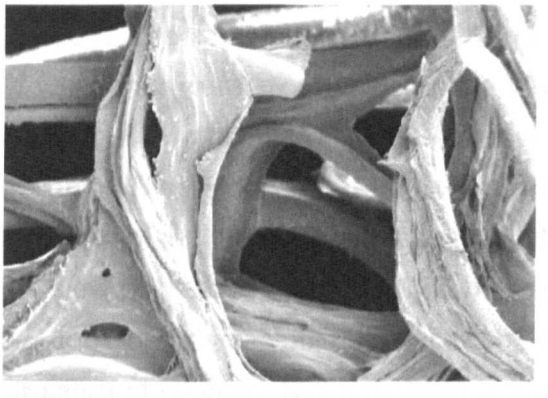

Figure 2.9: SEM image of white hair degraded following treatment with sodium sulfide for 3 hours (mag. x200, 5.0kV).

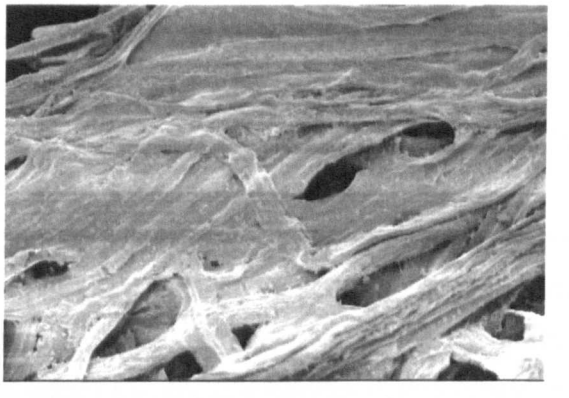

Figure 2.10: SEM image of white hair degraded following 5 hours treatment with sodium sulfide (mag. x200, 5.0kV).

Whereas the brown and black hair appears to have been dissolved from the inside as demonstrated by the collapsed structures, they appear to retain some structural integrity compared to the white hair which appears disintegrated (Figure 2.9).

The extent of damage to the white hair is supported by the high percentage of solubilised keratin nitrogen measured as total nitrogen by the TKN (Section 2.4.3.1, Figure 2.6). A comparison between brown hair and black hair (Figures 2.7 & 2.8) shows evidence of more cuticle damage to the black hair than brown hair within the same time scale. After five hours under the reducing conditions of sodium sulphide, the SEM image of the white hair shows evidence of further severe structural damage (Figure 2.10) compared to the brown and black hair samples under the same conditions (Figures 2.11 & 2.12). Although the brown and black hairs were equally damaged at this point, it was more of a fragmentation, whereas the white hair appeared gelatinised and sticky, indicating the sample had undergone severe structural modification.

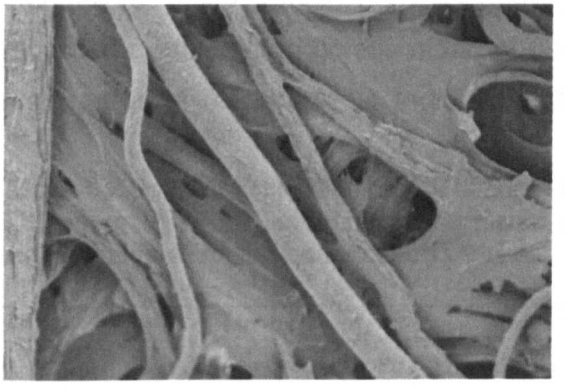

Figure 2.11: SEM image of damaged brown hair following 5 hour treatment with sodium sulfide (mag. x200, 5.0kV).

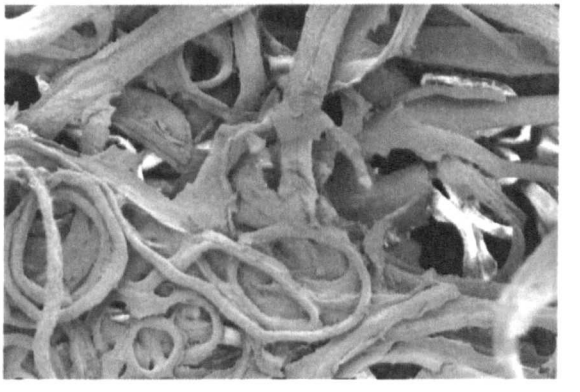

Figure 2.12: SEM image of damaged black hair following 5 hours treatment with sodium sulfide (mag. x200, 5.0kV).

These representative microscopy (SEM) images provide evidence that the cortical proteins of white hair were more vulnerable and easily solubilised under the reducing conditions of sodium sulfide compared to the cortical proteins of brown and black hair, which contains melanin. The evidence, so far provided (Section 2.4.1.4), indicates that melanin does not form part of the molecular structural architecture of hair. Therefore, it may be suggested that the slow degradation of melanised hair samples (black and brown) by sodium sulphide may be due to the insoluble character of melanin, and more particularly, larger molecular structure of melanin. Since melanin is tightly held within the cortical keratin matrix (Dawber, 1996; Ito and Jimbow, 1983; Liu *et al.*, 2003; Riley, 1997) their larger molecular size may have acted as a barrier to penetration by sodium sulphide, and thus causing limited accessibility of the intra-chain disulfide crosslinks of the cortical proteins by sodium sulphide.

2.4.3.2 Laboratory studies using sodium sulfide

2.4.3.2.1 Thermal analysis of sodium sulfide-treated hair samples

The thermal transition temperatures of the sodium sulfide-treated samples studied are summarised in Table 2.4. The data represent mean values of the fundamental parameters derived from the thermal plots of the peak glass transition temperature (T_g), enthalpy of glass transition (ΔH_g), peak denaturation temperature (T_D), and enthalpy of denaturation (ΔH_D). The mean values of glass transition temperature (T_g) obtained from the area under the peak on the respective curves indicate that there was no impact on the glass transition temperature following the treatment. On the other hand, there was an impact on both enthalpy of glass transition ($\Delta H_{g,}$) and enthalpy of denaturation (ΔH_D). Both parameters recorded an overall arithmetic mean of 99 J/g and 0.6 J/g, respectively, compared with the mean value of 149 J/g ($\Delta H_{g,}$) and 5.2 J/g (ΔH_D) for the control samples (intact hair).

Table 2.4: Average thermal transition temperatures observed from the thermal plots measured by DSC (from 20 – 250°C at 5°C min^{-1}) for brown, black and white bovine hair following treatment with sodium sulfide for 1 – 5 hours at 20°C.

Hair sample	Time of treatment (hrs)	Peak 1 Transition temp. (°C)		Peak 2 Transition temp. (°C)	
		(T_g)*	(ΔH_g) J/g*	(T_D)*	(ΔH_D) J/g*
Brown (1)	1	147 ± 2	131 ± 4	226 ± 1	1.3 ± 0.8
(2)	2	146 ± 2	113 ± 5	224 ± 1	0.2 ± 0.3
(3)	3	147 ± 3	105 ± 5	222 ± 2	0.2 ± 0.1
(4)	5	146 ± 4	64 ± 12	ND	ND
Black (1)	1	147 ± 3	129 ± 5	224 ± 2	1.6 ± 1.0
(2)	2	145 ± 3	114 ± 10	223 ± 1	0.3 ± 0.1
(3)	3	145 ± 2	100 ± 13	222 ± 1	0.2 ± 0.2
(4)	5	147 ± 3	78 ± 8	ND	ND
White (1)	1	146 ± 2	120 ± 6	224 ± 1	1.1 ± 0.5
(2)	2	147 ± 4	83 ± 4	220 ± 1	0.06 ± 0.02
(3)	3	143 ± 3	77 ± 6	ND	ND
(4)	5	146 ± 3	72 ± 11	ND	ND

ND = peak not defined. *Mean of six duplicates ± confidence interval.

A comparison between the samples treated with sodium sulfide at different times (1 – 5 hrs) indicated that white hair consistently recorded low values of enthalpy of glass transition (($\Delta H_{g,}$) and enthalpy of denaturation (ΔH_D) as compared to values for black and brown hair (Table 2.4). The results suggest that the structural stability of white hair was more rapidly modified than black and brown hair samples. This correlates with the results from the percentage of solubilised keratin nitrogen (Figure 2.6, Section 2.4.3.1) and evidence from the microscopic (SEM) physical structure changes observed (Section 2.4.3.1.1). Generally, it was also recognisable that there was a gradual decrease in the denaturation temperature with progressive time of treatment. This is consistent with an explanation by Wortmann *et al.* (2008), which states that repeated treatments with a reducing agent leads to a systematic decrease of denaturation temperature (T_D) as well as enthalpy for the glass transition (ΔH_g).

Figures 2.13 and 2.14 show thermal curves when major thermal events between the samples were observed and compared against the control sample. Figure 2.13 show thermal characteristics and variability between the samples after 3 hours treatment. Whilst black and brown hair each show higher glass transition temperatures of 145 and 147°C, the observed peak for the white hair sample was 143°C, suggesting some modification of its thermal properties. At the same time black and brown hair appears to show a bump at the denaturation temperature peaks (arrows), whereas the white hair seems to have none.

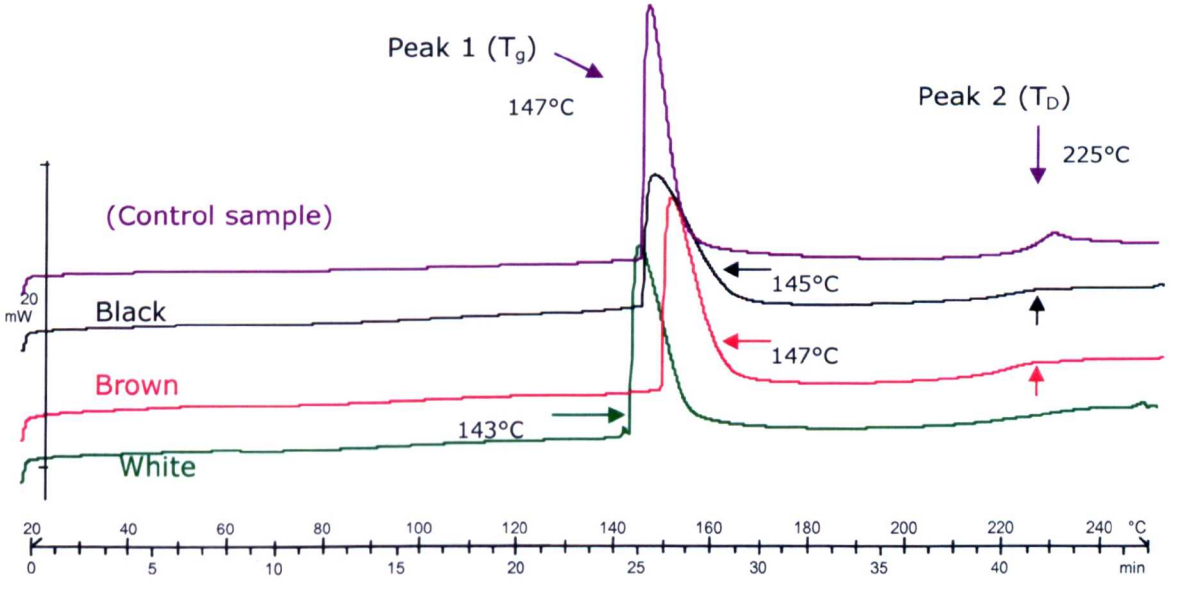

Figure 2.13: Thermal transition temperature curves for black, brown, and white bovine hair samples treated with sodium sulfide for three hours. Black and brown hair samples exhibiting small endothermic peaks (arrow) at 222°C. The thermal curve for intact hair sample is used as a control. The values are mean temperatures of samples heated from 20 – 250°C at 5°C min^{-1}.

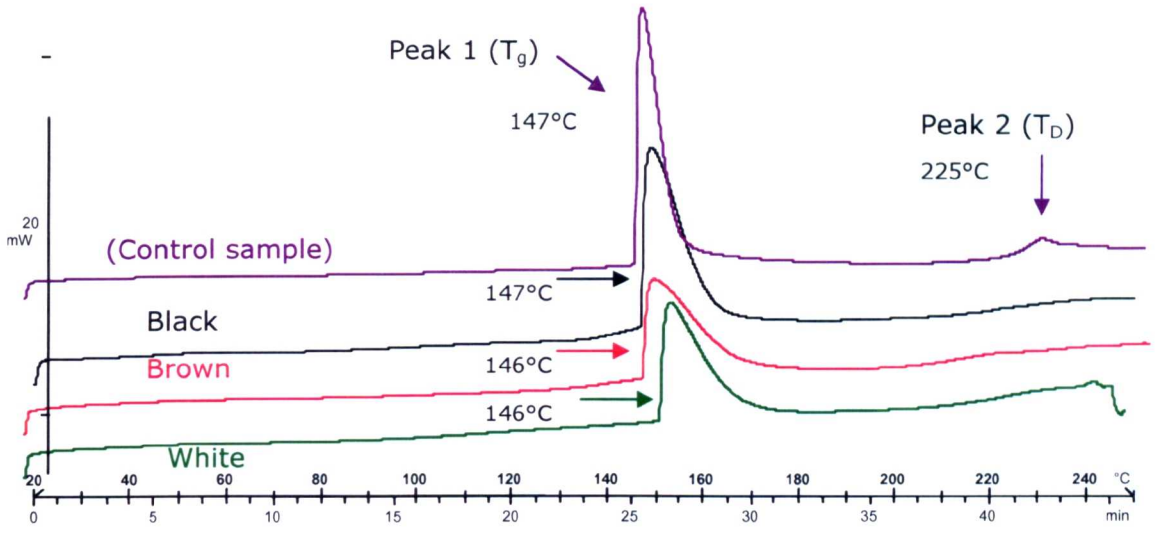

Figure 2.14: Thermal transition temperature curves for black, brown and white bovine hair treated with sodium sulfide for five hours. The thermal curve for intact hair sample is used as a control. The values are mean temperatures of samples heated from 20 – 250°C at 5°C min^{-1}.

Figure 2.14 shows the effects of sodium sulfide on the denaturation temperature (T_D) of the samples following a 5 hour treatment. While all the samples still retained a glass transition temperature peak at 146, there was no visible peak for the denaturation temperature. The results suggest a major alteration of the structures responsible for hair stability (*i.e.* destruction of disulfide crosslinks) in all the samples after 5 hours under reducing conditions of sodium sulfide.

2.4.3.3 Chemical pretreatments

The results of the solubilised hair proteins following chemical pretreatments are shown in Figure 2.15. The results show the protein content of the soluble fraction as mg/ml, determined by the method of Lowry *et al.*, (1951). The average total protein content in 0.05 g of the dry hair sample was estimated to be 47 mg. This represented 94% dry weight of the sample. The average concentration of soluble protein following treatments with guanidine hydrochloride and urea was 0.055 mg/ml and 0.049 mg/ml, respectively, representing 0.12% and 0.1% of total hair proteins. At the same period the average concentration of soluble protein detected following treatment of hair with sodium hydroxide, (NaOH), was 0.61 mg/ml (1.3% of hair proteins), potassium thioglycolate, (KTG), 0.66 mg/ml (1.4% of hair proteins), and a combination of sodium hydroxide and potassium thioglycolate, (NaOHKTG), 1.72 mg/ml (3.7% of hair proteins), respectively.

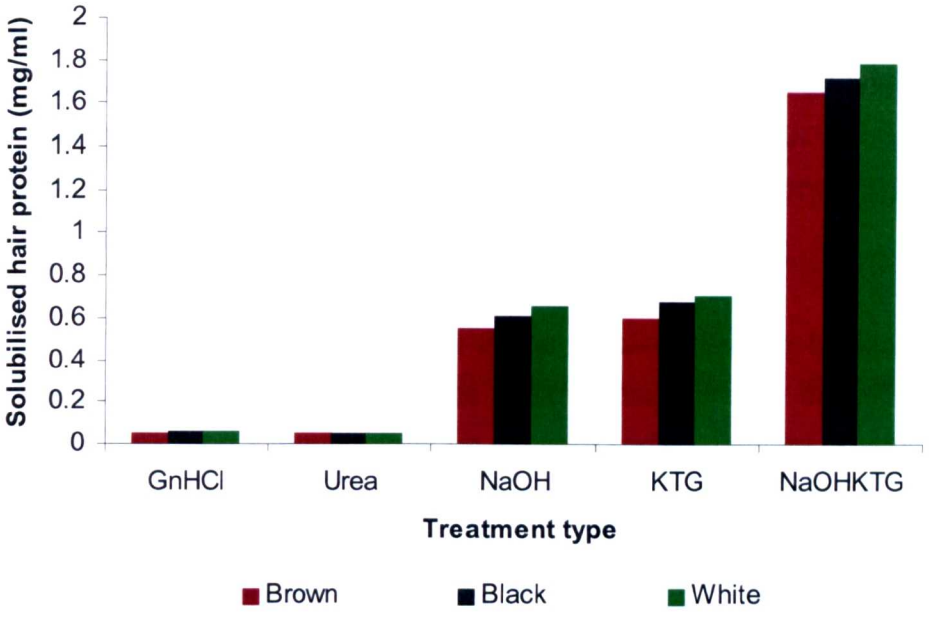

Figure 2.15: Comparison of the concentration of solubilised hair protein following pretreatment with 6M guanidine hydrochloride (GnHCl), 8M urea (Urea), 0.1M sodium hydroxide (NaOH), 0.1M potassium thioglycolate (KTG), and a combination of 0.1M sodium hydroxide and potassium thioglycolate (NaOHKTG) for one hour at 40°C.

Based on the results of the protein concentration, it may be concluded that treatment with guanidine hydrochloride and urea resulted in very slow modification of the hair. The possible explanation to this may due to the mechanism that these two reagents denature proteins, which is by way of interacting with the hydrogen bonds and hydrophobic groups, which are the weaker stabilising forces in keratin. A comparison of the mean values of soluble proteins due to NaOH and KTG treatments show there was no significant difference between the two. The results of the soluble protein measurements show that under the experimental conditions the efficiency of chemical modification was better and faster with the combined treatment employing alkali and potassium thioglycolate (NaOHKTG).

By comparing the results from thermal analysis (Section 2.4.3.3.1) and microscopy images (SEM) of the physical structure changes (Section 2.4.3.3.2.3), a correlation of the effects of the combined treatment provided evidence which indicates that by using a reducing agent to cleave the disulfide bonds, and an alkali to target the non-keratinous proteins, the hydrolysis and disintegration of the fibre was enhanced. This may be explained from the denaturing mechanisms of the two degrading agents as follows: (1) reducing agent specifically targets the disulfide (S–S) bonds through a nucleophilic displacement mechanism (Windus and Showell, 1968). The disulfide bonds are the main structural crosslinks that allow conformational stability of the folded three-dimensional structure of the protein molecule. The

destruction of these bonds lead to loss of strength and stability of the molecule (Feughelman, 2002; Fraser *et al.*, 1972; Mathews, 1954; Robinson, 1976), and (2) alkali preferentially attacks non-keratinous proteins, but may hydrolyse disulfide bonds by forming sulfhydryl groups at pH 10 and above. Other non-covalent bonds such as hydrogen bonds and salt-linkages are also degraded (Baumann, 1979; Goddard and Michaelis, 1935; Mathews, 1954; Merril, 1956).

Further more the effect of a reducing agent in the reaction may be to attack the highly crosslinked disulfide bonds of the hair cuticle, which initially breaks the diffusion barrier, since the cuticular cells form a tough barrier on the fibre surface (Stoves, 1948). This allows penetration of the alkali (NaOH) to attack the less crosslinked cell membrane, which is also responsible for diffusion of molecule into the fibre (Baumann, 1979). The attack on the cell membrane is followed by degradation of the non-keratinous materials within the cortex such as the orthocortex and cytoplasmic remnants, whilst the reducing agent (KTG) cleaves the keratin disulfide bonds. The combined effect of reduction and alkali hydrolysis brought about physicochemical transformation of the fibre more rapidly compared to the other treatments.

2.4.3.3.1 Thermal analysis of chemically pretreated hair samples

Results of the thermal analysis of chemically pretreated bovine hair samples are summarised in Table 2.5. The data presented are only from those treatments which have had major effects on the thermal properties. The results show that even though there were variations between the samples in the values obtained there was a decrease in the transition enthalpy, ΔH_g, the denaturation temperature T_D and enthalpy of denaturation, ΔH_D, in all the treatments. The variation between the samples may be ascribed to the different methods by which each treatment impacted on hair keratins.

Table 2.5: Average thermal transition temperatures observed from the thermal plots as measured by DSC (from 20 – 50°C at 5°C min^{-1}) for brown, black and white bovine hair following chemical pretreatments for 1 hour at 40°C.

Treatment	Hair sample	Transition temp. (°C) (T_g)	(ΔH_g) J/g	Transition temp. (°C) (T_D)	(ΔH_D) J/g
NaOH	Brown	145 ± 3	136 ± 4	222 ± 3	1.1 ± 0.8
	Black	143 ± 6	98 ± 7	218 ± 4	1.2 ± 0.8
	White	141 ± 4	91 ± 6	219 ± 3	1.0 ± 0.7
KTG	Brown	143 ± 2	123 ± 4	223 ± 2	1.0 ± 0.6
	Black	141 ± 2	123 ± 6	223 ± 1	1.4 ± 1.0
	White	143 ± 1	118 ± 3	223 ± 1	0.5 ± 0.5
NaOHKTG	Brown	140 ± 2	109 ± 6	ND	ND
	Black	141 ± 4	88 ± 3	ND	ND
	White	137 ± 3	84 ± 6	ND	ND

ND = peak not defined. *Mean of six duplicates ± confidence interval.

By comparing the treatments, a NaOHKTG pretreatment method was found to have a significant effect on thermal properties of hair. Within the experimental conditions, the treatment caused a decrease in glass transition temperature (T_g,) by an average of 8°C lower than for the intact hair (control samples). Similarly, the enthalpy for the glass transition (ΔH_g) decreased by 37% from 149 J/g for the intact hair (control) to 94 J/g. Figure 2.19 represent thermal curves illustrating the effects of NaOHKTG treatment on denaturation thermal peaks under the experimental conditions. There was no visible peak observed at the region for denaturation temperature (Figure 2.19), indicating major physicochemical changes may have occurred in the sample.

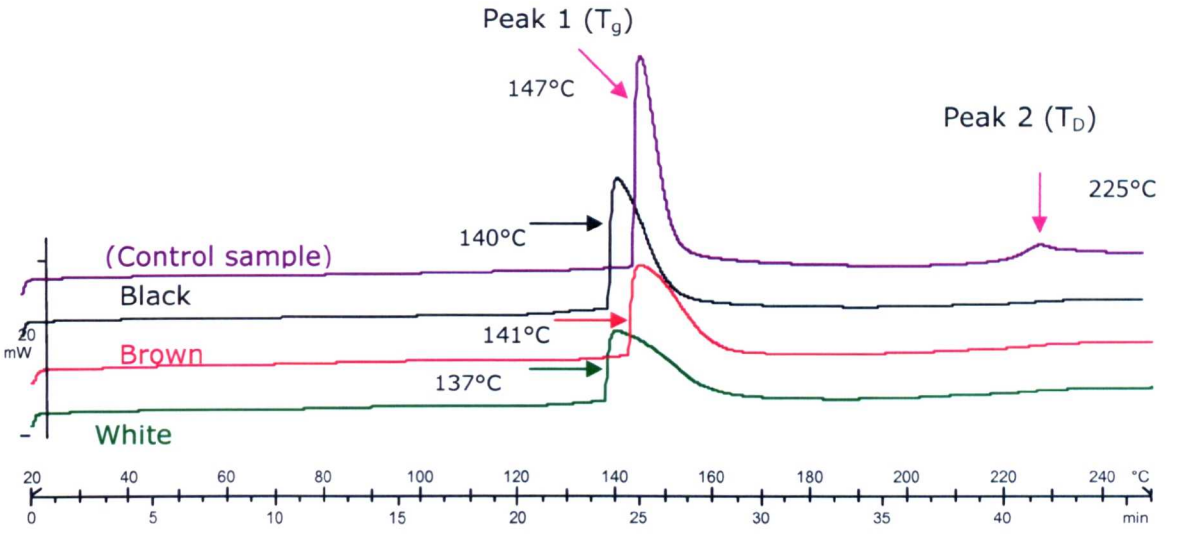

Figure 2.16: Thermal transition temperature curves for black, brown and white bovine hair treated with sodium hydroxide plus potassium thioglycolate (NaOHKTG, treatment) for one hour at 40°C. The thermal curve for intact hair sample is used as a control. Values shown are mean temperatures following heating from 20 – 50°C at 5°C min^{-1}.

2.4.3.3.2 Structural damage assessment

2.4.3.3.2.1 Effect of treatment with alkali (sodium hydroxide)

Figures 2.17 – 2.19 show microscopy images (SEM) of hair samples subjected to a 0.1 M sodium hydroxide pretreatment for one hour. The structural changes of the treated samples were compared with the structural features of intact hair, non-treated samples (Figures 2.2 & 2.3, Section 2.4.1.1). There was evidence of selective degradation, specifically targeting the cortical macrofibrils, as evidenced by the collapsed structure of the brown hair and partial degradation of black hair sample (Figures 2.17 & 2.18).

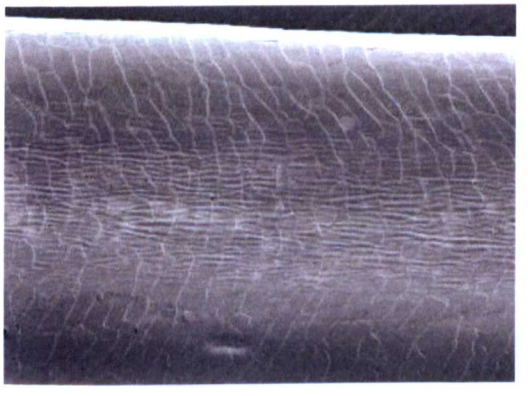

Figure 2.17: SEM image of brown hair illustrating the collapsed hair shaft following 1 hr treatment with sodium hydroxide (mag. x1.0, 5.0kV).

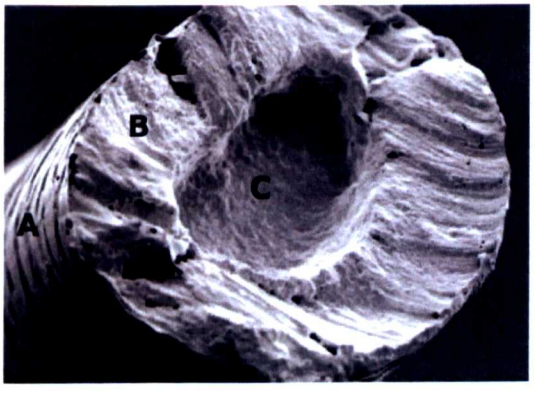

Figure 2.18: SEM image of black hair shaft illustrating partial damage to the cortex (B), and medulla (C) following 1 hr treatment with sodium hydroxide (mag. x1.0, 5.0kV).

There was also evidence of partial degradation of the medulla, observed on both the black and white hair samples (Figures 2.18 & 2.19). The cuticle was largely left intact, indicating the inability of the hydroxyl ions to cleave the disulfide bonds of the cuticle proteins.

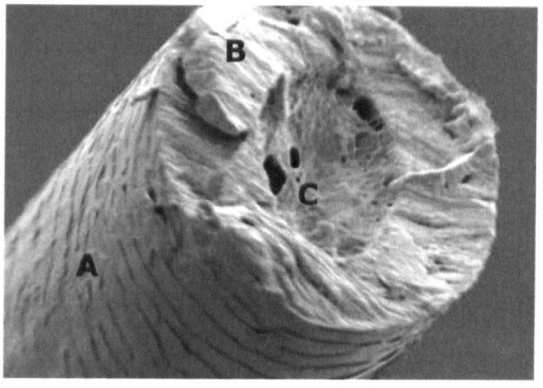

Figure 2.19 SEM image of white hair shaft illustrating partial damage to the medulla (C) following 1 hr treatment with sodium hydroxide (mag. x1.0, 5.0kV).

The results of the microscopy (SEM) images demonstrate that the preferential breakdown of hair using an alkali (NaOH) was through the dissolution of the non-keratin proteins. This is consistent with literature reviews on the effect of hydroxide ions on the structural components of hair (Addy *et al.*, 2001; El Baba *et al*, 2000; Stoves, 1948). Although the mild treatment caused minimal damage to the physical structure within the time scale of the experiment, it was evident that alkali treatment had two effects on hair: (1) chemical modification of the structure, as measured by the concentration of solubilised protein, and (2) physical structural modification, represented by the microscopy (SEM) images.

2.4.3.3.2.2 Effect of treatment with potassium thioglycolate

Thioglycolates have been used to modify keratin structures in hair or wool fibres (Burmeister and Rumson, 1995; Manuszak *et al.*, 1996). The microscopy (SEM) images of the modified physical structure of bovine hair during treatment with a reducing agent, potassium thioglycolate, are represented by Figures 2.20 – 2.22. The microscopic observations show the reaction was mainly confined on the surface of the fibre, as illustrated by the damage to the cuticle. The extensive degradation of the cuticle suggests that the disulfide crosslinks that are primarily responsible for the stability of these cells are being degraded. The observed structural changes further demonstrate preferential degradation of the hair structure according to the chemical properties of the components. The significance of this is that the treatment breaks the diffusion barrier, and weakens the structure by cleaving inter

and intra-chain covalent disulfide crosslinks which may subsequently enhance enzymatic digestion (Fontana and Gross, 1986).

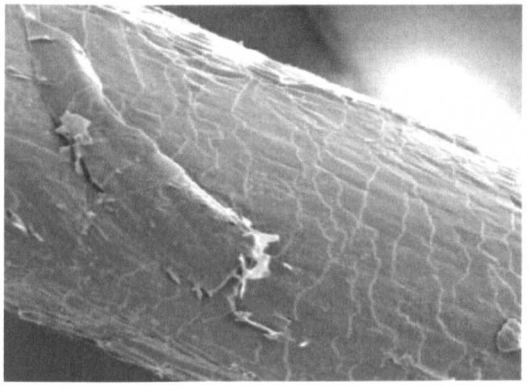

Figure 2.20: SEM image of brown hair shaft illustrating damage to the cuticle following 1 hr treatment with potassium thioglycolate (mag. x1.0, 5.0kV).

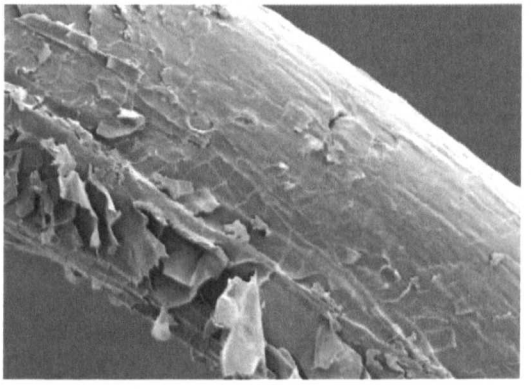

Figure 2.21: SEM image of black hair shaft illustrating damage to the cuticle following 1 hr treatment with potassium thioglycolate (mag. x1.0, 5.0kV).

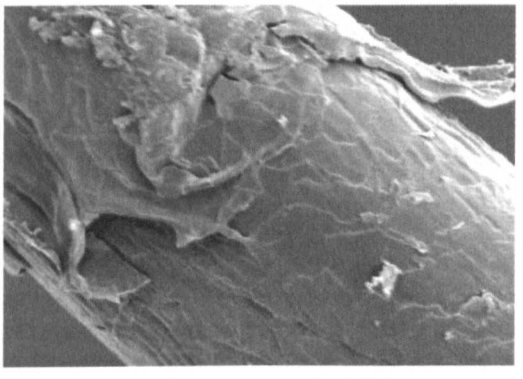

Figure 2.22: SEM image of white hair shaft illustrating damage to the cuticle following 1 hr treatment with potassium thioglycolate (mag. x1.0, 5.0kV).

2.4.3.3.2.3 Effect treatment with potassium thioglycolate plus sodium hydroxide.

Microscopy micrographs (SEM) of bovine hair samples treated with 0.1 M potassium thioglycolate and 0.1M sodium hydroxide are shown in Figures 2.23 – 2.25. The microscopy images show enhanced structural damage, particularly the cortical microfibrils and medulla. While the cuticle was not dissolved within the same time scale, its structural integrity appears to be weakened from the effect of a reducing agent and the dissolution of the cortex. A visual comparison of the microscopy (SEM) images of the physical structure modification of the hair samples indicates that the extent of structural damage was similar (Figures 2.23 – 2.25).

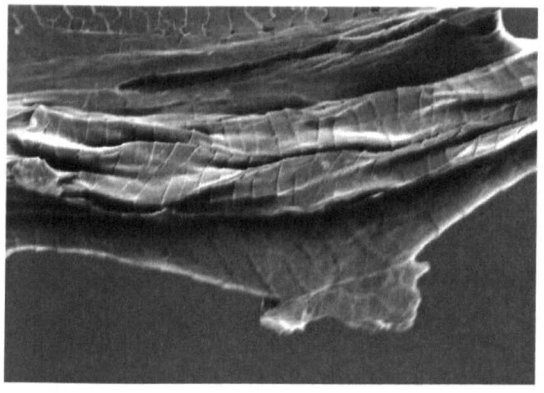

Figure 2.23: SEM image of brown hair illustrating damage to the hair shaft following 1 hr treatment with potassium thioglycolate & sodium hydroxide (mag. X1.0, 5.0kV).

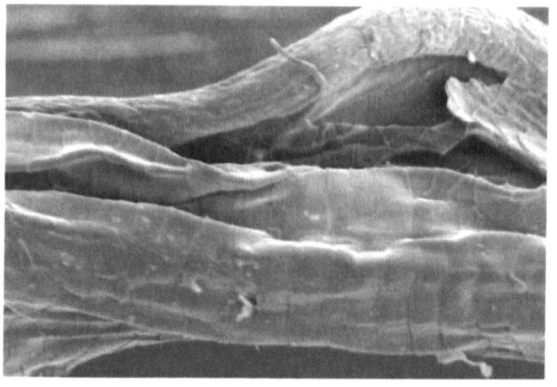

Figure 2.24: SEM image of black hair illustrating damage to the hair shaft following 1 hr treatment with potassium thioglycolate & sodium hydroxide (mag. x1.0, 5.0kV).

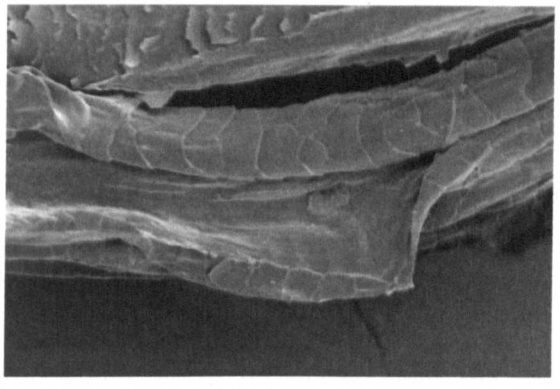

Figure 2.25: SEM image of white hair illustrating damage to the hair shaft following 1 hr treatment with potassium thioglycolate & sodium hydroxide (mag. x1.0, 5.0kV).

From the results of the concentration of soluble protein and microscopy images of physical structure modification, there is evidence that the application of a combination of a reducing agent and alkali as a single pretreatment enhanced structural breakdown of hair than when applied separately as individual treatments. The selective breakdown of the hair components may be explained in terms of their differences in the proportion of disulfide crosslinks. From the observed mechanism of chemical breakdown of hair using a reducing agent and alkali, it is apparent that an effective treatment of hair, which may lead to the disintegration of the structure, must incorporate a keratin degrading agent and protein hydrolytic agent to enhance the breakdown of non-keratin components.

The following chemically pretreated (NaOHKTG pretreated) hair samples were, subsequently, used as a representative of pretreated hair substrates for enzymatic digestion as presented in Chapter 3 of this thesis.

2.4.3.3.2.4 Effect treatment with urea and guanidine respectively.

Pretreatment of hair samples with urea and guanidine did not affect the thermal properties and no physical structure changes were observed within the time scale of the experimental conditions. This may be explained by the possible nature of the chemical interaction of urea and guanidine with proteins. The molecular denaturation mechanism is via interaction with hydrogen bonds and hydrophobic forces (Alonso and Dill, 1991; Frank and Franks, 1968; Shellman, 1987; Wallqvit *et al.*, 1998). In addition, due to the highly resistant cuticular layer, it is possible that these reagents were unable to penetrate the hair thus limiting their ability to cause damage.

2.4.4 Summary of the chapter

- Analyses of brown, black and white bovine hair samples (dry weight) showed the average of extractable lipids, ash, and nitrogen content to be 0.43% (w/w), 2.1% (w/w), and 15.3% (w/w), respectively. Black hair recorded a higher value of ash content (3.6%) than brown hair (1.6%), and white hair (1.2%). The variability in ash content may be attributed to environmental and nutritional factors, and possibly melanin content.

- An analysis by electrophoretic technique resolved the molecular weights of bovine brown, black and white hair keratin proteins in the range of 48kD – 59kD. Whereas the thermo-analytical properties by Differential Scanning Calorimetry show the average values of the glass transition temperature and denaturation temperature of bovine hair keratins to be 147°C and 225°C, respectively. The results show that the constituent proteins and thermal properties of brown, black and white bovine hair are the same. This suggests that melanin may not constitute part of the molecular architectural structure of hair, which may be supported by the fact that melanin is not a protein (Rodgers and Dean, 2000).

- Analyses of solubilised hair keratin nitrogen by Total Kjeldahl Nitrogen indicated that the solubilisation of white hair was faster than pigmented bovine hair (black and brown) under reducing conditions of sodium sulfide. This was supported by the observed microscopic (SEM) physical changes and changes in the thermal properties. The treatment decreased thermal stability of white hair than pigmented hair after treatment for 2 hrs and 3 hrs, respectively. However, there was no significant difference in thermal properties of the samples after treatment for 5 hrs.

- Under the experimental conditions the denaturation temperature and disintegration of the physical structure of hair was enhanced following pretreatment with a mixture of alkali (NaOH) and potassium thioglycolate. The treatment mixture solubilised 3.6% of hair keratin within the experimental period. The enhanced reaction may be attributed to the cooperative effect of temperature, hydrolytic action of alkali on non-keratinous proteins, and reducing agent acting on the highly crosslinked disulfide bonds; the reagents acting selectively on different hair components.

- The observed mechanism of chemical degradation of hair allow us to infer that, to enhance the breakdown of hair, requires the presence of keratinolytic agent to break the disulfide bonds and a hydrolytic agent to accelerate the breakdown of the non-keratinised proteins. This forms the basis of the biochemical approach carried out in chapter 3.

CHAPTER 3

ENZYMATIC DEGRADATION OF BOVINE HAIR

CHAPTER 3

3. INTRODUCTION

3.1 Industrial enzymes

The application of biotechnological processes for clean industrial products is recognised as a cost-effective and eco-efficient technology that contributes to sustainable development, according to the Organisation for Economic Cooperation and Development (OECD, 2001). At the centre of this technology are biological catalysts, (industrial enzymes), that are receiving much of the attention for industrial purposes because they are efficient, specific and selective, thus offering cleaner products and processes than non-biological alternatives (Bull *et al.*, 1999). The versatility of this field of biotechnology is providing novel opportunities for sustainable production of new products and services. Consequently, many industries that previously never considered biological processes as impacting on their business, are exploring the possibility of doing so (Bull *et al.*, 1999; OECD, 1998; Gavrilescu and Chisti, 2005; Marrs *et al.*, 1999). In the European Union, biotechnology is considered as having great potential and benefits to the member states in terms of achieving major policy goals such as economic growth, job creation, public health, environmental protection and sustainable development (Zika *et al.* 2007).

The global demand for industrial enzymes is on the rise. According to a technical market research from Business Communications Company, Inc., new and emerging applications are helping drive the demand for enzymes and the industry is equally responding with innovative products. The global market for industrial enzymes increased from $2.2 billion in 2006 to nearly $2.3 billion in 2007 and is expected to rise at a compound annual growth rate of 4% to $2.7 billion by 2012 (Thakore, 2008).

Following increasingly stringent standards on the discharge of wastes into the environment considerable research initiatives have been conducted to explore new possibilities offered by enzymes in waste treatment (Karam and Nicell, 1997). Three main reasons have been suggested for the increasing interest in the use of enzymes in waste management: (1) there is an increase in the rate of introduction of xenobiotics and recalcitrant organic pollutants into the environment, making it difficult to achieve the required level of efficiency of removal by using the conventional chemical and biological processes. There is, therefore, a need for the development of alternative treatment methods that are faster, cheaper, more reliable, and

easy to implement than the present processes; (2) there is increasing awareness that enzymes can be used to target specific pollutants for treatment; and (3) recent biotechnological advances in the production of cheaper and more readily available enzymes through better isolation and purification procedures (Karam and Nicell, 1997). The majority of the industrial enzymes in use are hydrolytic in action, being used for the degradation/depolymerisation of natural substances (Godfrey and West, 1996; Kirk *et al.*, 2002).

3.2 General properties and characteristics of enzymes

Enzymes are protein molecules that act as highly efficient catalysts in biochemical reactions (Berg *et al.*, 2001; Enzymes Technical Association, 2002). They are naturally produced in nearly every organism and plant in order to facilitate metabolic and cellular processes. The principles of enzyme catalysis is based on the enzyme providing a reaction pathway with a transition state whose free energy of activation (ΔG) is lower than that of the non-catalysed reactions (Berg *et al.*, 2001; Uhlig, 1998; Voet *et al.*, 2008).

Enzymes are broadly classified into six groups according to the type of reaction catalysed. This includes hydrolytic enzymes (esterases, proteases), transferases, oxidoreductases, isomerases, lyases, and ligases (Palmer, 1981). Proteases, which catalyse the hydrolysis of peptide bonds in proteins and ester bonds, are the most dominant and diverse group of enzymes. The catalytic mechanisms and active sites vary widely among different proteases, which form the basis of their classification as serine, cysteine, aspartic, glutamic, threonine, or metalloproteases (Lopez-Otin and Bond, 2008; Rao *et al.*, 1998), and as endopeptidases (targets internal peptide bonds away from the amino and carboxy termini of their substrates) or exopeptidases (cleaves the peptide bonds from either end of the amino and carboxy termini of their substrates) (Barret *et al.*, 1998; Beynon and Bond, 2001; Rao *et al.*, 1998). Proteases may also be classified on the basis of the pH range over which they are active as, acid, neutral, or alkaline proteases, or on the basis of their ability to hydrolyse specific proteins such as, keratinase (acting on keratin substrates), collagenase (acting on collagen), and elastase (acting on elastin) (North, 1982).

3.2.1 Specificity of enzymes

The characteristic feature of enzymes is the specificity of their action. While some enzymes are highly specific for only one substrate, others exhibit multiple specificity, meaning, they may act on several different, though closely related, substrates to catalyse a reaction

(Palmer, 1981; Tabatabai and Dick, 2002; Voet *et al.*, 2008). Proteases exhibit diverse specificity. Some show marked specificity toward peptide bonds of a single protein, but most proteases are relatively non-specific, having the capability to target multiple substrates (Lopez-Otin and Bond, 2008). The general formula that describes the interaction between an enzyme and its substrate may be expressed as shown in Scheme 3.1.

Scheme 3.1: An enzyme substrate interaction (Palmer, 1981)

3.3 An approach to enhance enzymatic hydrolysis of hair

In the previous chapter the breakdown of hair was found to be enhanced by a process involving a combination of a reducing agent as a keratinolytic agent acting on the highly keratinised components, and an alkali as a hydrolytic agent targeting the soft keratins and non-keratinous components. In this chapter a laboratory-scale study of a biochemical (enzymatic) system capable of causing a complete breakdown of hair is proposed. The proposed biochemical approach is, therefore, derived from the observed mechanism of chemical breakdown of hair components. Thus the proposed work involves the concept of using a combined protease assay with different substrate specificities, *i.e.* a keratinase as a keratinolytic agent with the specificity for the highly keratinised components hair, and a general protease as hydrolytic agent to act on the non-keratinous components. Although the two enzymes belong to the group of proteases, but due to the nature of the differences in their substrate specificity; in this study, the term keratinase will be used for keratin degrading enzyme, while protease is used to mean non-keratin degrading enzyme.

The principal aim of this study was to exploit the different properties of proteases and the biochemical differences of the hair components to formulate an enhanced natural biochemical (enzymatic) breakdown process. This Chapter will also discuss the possible mechanism of enzymatic breakdown of hair, relative to its structural components. The key objectives are:

- To evaluate the relative rates of enzymatic breakdown of native hair by (i) keratinase, (ii) protease, and (iii) combined keratinase plus protease.
- To optimise the parameters for the enzymatic degradation of hair (pH and temperature).
- To evaluate the effects of chemical pretreatment on the enzymatic breakdown of hair.

- To analyse the physicochemical changes.

The outcome of the proposed laboratory studies will be used to identify a cheaper source of the biochemical mixture.

3.4 Materials and methods

3.4.1 Sample preparation

Bovine hair samples (brown, black and white) were prepared as described in Section 2.6.1.

3.4.2 Enzymes and other reagents

Commercial enzyme proteinase K, from Sigma Aldrich (UK) was used as a source of keratinase (Ebeling *et al.*, 1974; Sweeney and Walker, 1993). Trypsin from Sigma Aldrich (UK) was used as a source of protease (Wilkinson, 1986). 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) solution (Sigma Aldrich, UK) was used as a serine protease inhibitor (Conseil *et al.*, 1999). Analytical reagent, calcium chloride ($CaCl_2$) was obtained from Sigma Aldrich, UK. Keratin azure (Sigma Aldrich, UK) was used as an assay for keratinase activity. Hide Powder-Remazol Brilliant Blue (Fluka, UK) was used as an assay for proteolytic activity. All buffer solutions were prepared with deionised water using analytical grade reagents.

3.4.3 Enzymatic assays

Two different chromogenic substrates were used for the enzyme assays: (1) keratin azure: - this is a fibrous substrate to which a blue dye, Remazol Brilliant Blue, has been covalently bound to hair, and (2) Hide Powder-Remazol Brilliant Blue: - the substrate is derived from hide powder and labelled covalently with Remazol Brilliant Blue dye. In both cases, as the peptide bonds of the protein substrate are hydrolysed the bound dye is solubilised, which can then be measured spectrophotometrically as a measure of degradation (Rinderknecht *et al.*, 1968; Wainwright, 1982).

3.4.3.1 Optimisation of assay parameters

3.4.3.1.1 Enzyme concentration

To determine the appropriate enzyme concentration suitable for assay studies, proteinase K was initially studied in the range of 0.5 – 2 mg/ml, and trypsin, 1 – 3 mg/ml, with keratin azure as a substrate. Proteinase K, 1 mg/ml, and trypsin, 2 mg/ml, were the enzyme concentrations, respectively found appropriate for enzymatic studies.

3.4.3.1.2 Optimisation of pH

To determine optimum pH, the activity of the enzyme was studied using 50 mM Tris-HCl and 0.1 M ammonium bicarbonate buffer at a pH range 7.0 – 10, with keratin azure and Hide Powder-Remazol Brilliant Blue as substrates.

Although both enzymes were active in ammonium bicarbonate buffer, the buffer was not compatible with calcium chloride which was used as a cofactor for both enzymes (formation of calcium carbonate). Therefore, all the enzyme assays were subsequently carried out in Tris-HCl buffer.

3.4.3.1.3 Optimisation of temperature

To determine the temperature for optimum activity and stability each enzyme (proteinase K and trypsin) was studied by incubating at temperatures 37°C, 40°C, 50°C and 60°C.

3.4.3.1.4 pH: measurements were carried out using a SevenMulti electrochemical pH meter (Mettler-Toledo Ltd, UK)

3.4.3.2 Keratinase assay

Keratinolytic activity of proteinase K was tested using keratin azure as a substrate based on the method by Wainwright (1982). The reaction mixture comprising a final enzyme concentration of 1 mg/ml, in a 2 ml solution of 50 mM Tris-HCl buffer, pH 7.5, containing 20 mM $CaCl_2$ and 10 mg of keratin azure, was incubated at 37°C. The incubation was carried under constant agitation at 100 r.p.m in an orbital shaker (SI 50, Stuart Scientific, UK) for 1 hour. The assays were carried out in duplicate. A blank contained the substrate and buffer without the enzyme. The solubilised substrate was separated by centrifugation (Jouan GR20.22) at 5000 r.p.m at 4°C for 10 minutes. The absorbance of the supernatant was measured at 595 nm on a UV 2501PC spectrophotometer (Shimadzu, Japan). One unit of enzyme activity was defined as the amount of enzyme that catalysed the release of the dye to cause an increase in absorbance of 0.01 at 595 nm after 1 hour under the standard assay conditions (Wainwright, 1982).

3.4.3.3 Protease assay

Proteinase K and trypsin activity were assayed using Hide Powder-Remazol Brilliant Blue as a substrate (Bajorath *et al.*, 1988; Yamamoto and Sinohara, 1993). The substrate (10 mg) was incubated with the enzyme solution (3 ml) in 50 mM Tris-HCl buffer, pH 7.5, at 37°C for 1 hour. The assay procedure and subsequent treatment of the supernatant is as described in Section 3.4.3.2. The effect of pH and temperature were studied as described in Sections 3.4.3.1.2 and 3.4.3.1.3.

3.4.4 Enzymatic degradation of the keratin substrate

3.4.4.1 Enzymatic studies using keratin azure

Prior to enzymatic degradation of hair, the keratinolytic (proteinase K), and proteolytic (trypsin) degradation of keratin was studied using keratin azure as a substrate under the optimised conditions of pH, temperature and enzyme concentration. The purpose of the study was to compare the effects of using individual enzymes, and a combined enzyme (proteinase K plus trypsin) digestion of the chromogenic fibrous substrate. The intensity of the dye (absorbance) released was converted to units of activity, and used as a quantitative measure of the extent of hydrolysis according to Wainwright, (1982). The study was divided into three parts as follows: (1) the substrate, keratin azure (10 mg), was incubated with 2 ml solution of 1 mg/ml proteinase K in 0.1 M Tris-HCl buffer, pH 8.0, containing 20 mM $CaCl_2$ for 0 – 48 hrs at 40°C as described in Section 3.4.3.2. The supernatant was treated as described in Section 3.4.3.2, and the absorbance measured at 595 nm, (2) enzymatic digestion of keratin azure (10 mg) with a 2 mg/ml trypsin solution (2 ml) in 0.1 M Tris-HCl buffer, pH 8.0, containing 20 mM $CaCl_2$ at 40°C. The incubation and all subsequent procedures are as described in Section 3.4.3.2, and (3) enzymatic digestion of keratin azure (10 mg) with enzyme solution (2 ml) containing proteinase K (1 mg/ml) and trypsin (3 mg/ml) under the same conditions as described. All assays were carried out in duplicate.

3.4.4.2 Control sample

Unless otherwise mentioned all control samples in the experiments contained the substrate in assay buffer with 20 µl of 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), a serine protease inhibitor (Conseil *et al.*, 1999).

3.4.4.3 Keratinase degradation of hair

To determine the effect of enzyme (keratinase) on the degradation of hair; each sample, *i.e.* brown, black and white hair samples (10 mg) were incubated with an enzyme solution containing proteinase K (1 mg/ml) in 2 ml of 0.1 M Tris-HCl buffer, pH 8.0, with 20 mM $CaCl_2$ at 40°C under constant agitation as described in Section 3.4.3.2 for 0 – 72 hours. All experiments were carried out in duplicate. The control sample was treated as described in Section 3.4.4.2. The unsolubilised substrate was separated by centrifugation at 5000 r.p.m at 4°C for 10 minutes. The protein content was determined on the supernatant.

3.4.4.4 Trypsin degradation of hair

To determine the proteolytic effect on hair substrates, enzymatic digestion with trypsin (3 mg/ml) was carried out in 0.1 M Tris-HCl buffer pH 8.0 containing 20 mM $CaCl_2$ under similar conditions described above (Section 3.4.4.3).

3.4.4.5 Keratinase and trypsin degradation of hair

The purpose of this experiment was to determine the relative effects of using a mixture of proteinase and keratinase in the degradation of hair. Hair substrate (10 mg) was incubated at 40°C with an enzyme solution (2 ml) containing a combination of trypsin (3 mg/ml) and proteinase K (1 mg/ml), respectively, in 0.1 M Tris-HCl buffer, pH 8.0, containing 20 mM $CaCl_2$. The incubation was carried out under constant agitation as described in Section 3.4.3.2 for 0 – 72 hours. All experiments were carried out in duplicate. The control sample was treated as described in Section 3.4.4.2. The unsolubilised substrate was separated as described in Section 3.4.4.3. The concentration of hair protein solubilised was determined on the supernatant.

3.4.4.6 Effect of potassium thioglycolate and sodium hydroxide pretreatment on enzymatic degradation of bovine hair substrate

To determine the effects of chemical pretreatment on the improvement of enzymatic digestion of hair, the previously chemically (NaOHKTG, Chapter 2, Section 2.4.3.3.2.3) pretreated hair substrate was incubated with an enzyme solution (2 ml) containing a mixture of proteinase K (1 mg/ml) and trypsin (3 mg/ml), respectively, in 0.1 M Tris-HCl buffer, pH 8.0, containing 20 mM $CaCl_2$. The incubation was carried out in series of flasks at 40°C in an orbital shaker under constant agitation as described in Section 3.4.3.2, for 0 – 48 hours. The concentration of protein solubilised was determined at each incubation period.

3.4.4.7 Kinetic parameters

The kinetics of the enzyme catalysed reaction were determined by calculating the initial reaction rate, the initial velocity (V_0), by drawing a tangent to the linear part of the curve (Figure 3.1). The initial velocity (V_0) is defined by the slope of the tangent (Equation 3.1).

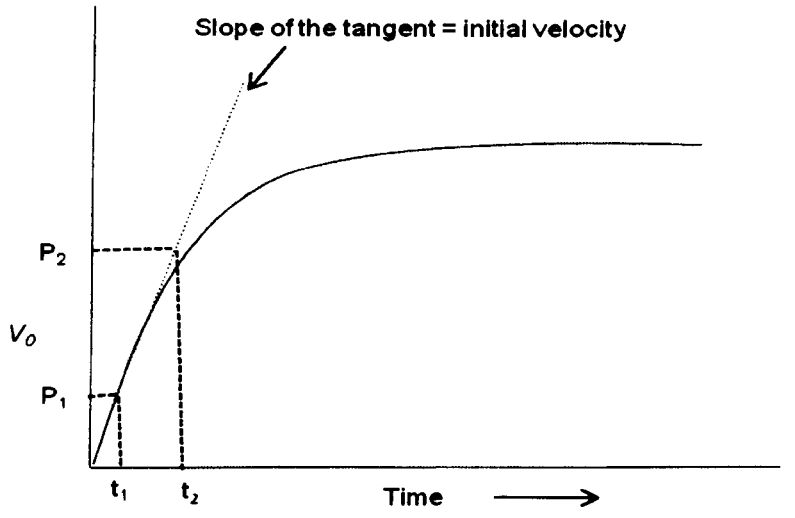

Figure 3.1: Typical curve for the calculation of the initial reaction rate (V_0) from the time dependent change in substrate concentration (P) of an enzyme catalysed reaction (Palmer, 1981).

$$V_0 = \frac{[P]_2 - [P]_1}{t_2 - t_1}$$ **Equation 3.1**

3.4.5 Protein determination

In order to monitor the chemical changes during enzymatic degradation, the concentration of hair protein solubilised was determined using the method of Lowry *et al.*, (1951) as described in Section 2.2.2.6.3 with bovine serum albumin (Sigma Aldrich, UK) as the standard. The absorbance was measured at 500 nm on a UV 2501PC spectrophotometer (Shimadzu, Japan).

3.4.6 Analysis of enzyme digested hair proteins using SDS-PAGE

To determine and compare the products of enzyme (keratinase) digestion, electrophoresis (SDS-PAGE) was used to characterise the molecular characteristics of the solubilised hair proteins. The SDS-PAGE was carried out according to the method by Laemmli (1970) using a 15% v/v resolving gel and a 4% v/v stacking gel as described in Section 2.2.2.6.4 (Chapter 2).

3.4.7 Scanning electron microscopy (SEM)

To monitor the progressive physical structure changes and assessment of the extent of damage to the substrate following enzymatic treatment, scanning electron microscopy examination (SEM) was carried out as described in Section 2.6.2.1.1.

3.4.8 Statistical analysis

For the enzymatic treatment experiments the mean values of the measured parameters were calculated from the duplicate runs and compared as described in Section 2.3.

3.5 Results and discussion

3.5.1 The effects of pH, temperature and substrate on enzyme activity

Figures 3.2 and 3.3 show the profiles of proteinase K and trypsin enzymes activity at different pHs and temperatures using keratin azure as a substrate. The pH and temperature of maximum activity for both enzymes was observed to be 8.0 and 40°C, respectively. This was expressed by increased intensity of soluble dye released from the substrate. From the activity profile, proteinase K exhibited an activity in a broad range of pH and temperature as reflected in Figures 3.2 and 3.3, respectively. In both cases the activity of proteinase K was greater than that of trypsin under the assay conditions demonstrating its effectiveness in breaking down the insoluble keratin substrate. This is in good agreement to other literature (Ebeling *et al.*, 1974). Although trypsin showed some keratin hydrolysing activity, the activity was significantly lower, ($p < 0.05$), compared to that of proteinase K. The activity of trypsin was observed to be further reduced at alkaline range of pH 10 and at a temperature of 60°C, indicating that the enzyme may have been denatured. The following results, therefore, established the optimal conditions for enzymatic activity as pH 8.0 and 40°C.

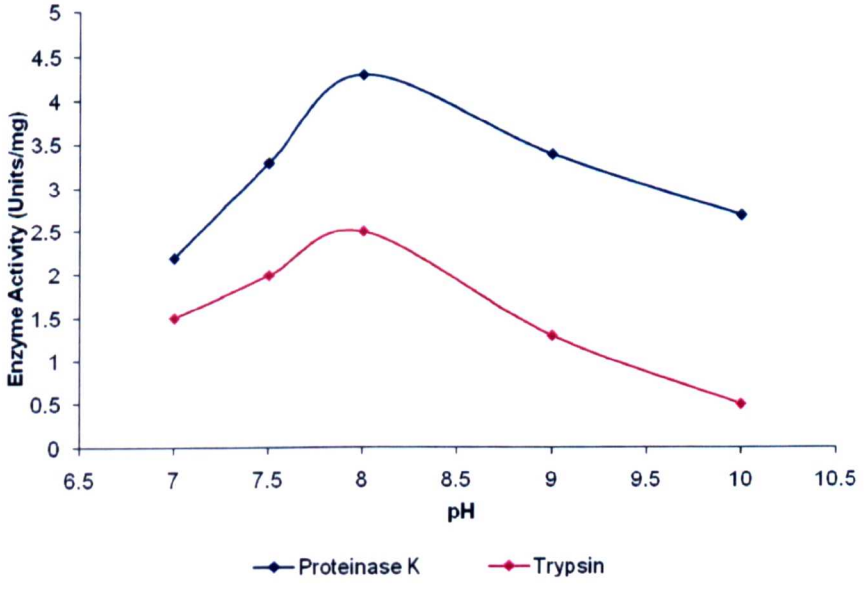

Figure 3.2: The keratinolytic activity of proteinase K and trypsin enzymes studied in the pH range of 7 - 10 with keratin azure as a substrate.

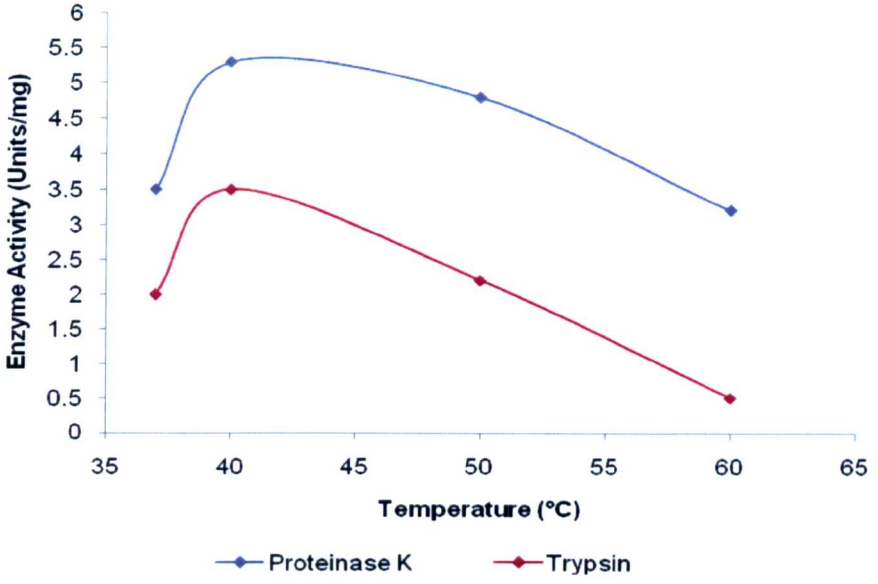

Figure 3.3: The keratinolytic activity of proteinase K and trypsin enzymes studied at pH 8.0 and temperature range of 37 - 60°C with keratin azure as a substrate.

Figure 3.4 illustrates the proteolytic activity profiles of proteinase K and trypsin enzymes with Hide Powder-Remazol Brilliant Blue as a substrate. Both enzymes exhibited higher activity in the hydrolysis of the hide powder substrate as compared with keratin azure. However, under the assay conditions, trypsin digestion significantly increased the release of the dye from the substrate measured by the UV spectrophotometer. This demonstrates a higher activity of trypsin as a protease than proteinase K. Within the experimental conditions it was observed that the activity of each enzyme increased as a function of time. Since trypsin is a highly specific enzyme (Wilkinson, 1986) the result further suggests the observed increase in tryptic activity is due to substrate specificity. On the other hand, the results of this study also show proteinase K as an enzyme of broad specificity (Ebeling *et al.*, 1974; Sweeney and Walker, 1993).

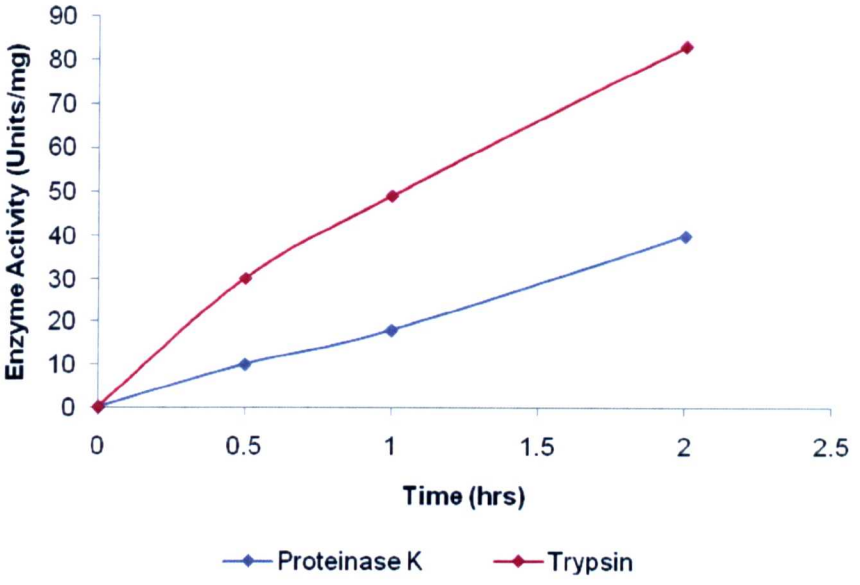

Figure 3.4: The proteolytic activity of proteinase K and trypsin enzymes studied at pH 8.0 and 40°C with Hide Powder-Remazol Brilliant Blue as a substrate.

3.5.2 Comparative enzymatic studies using keratin azure as a substrate

The results of enzymatic hydrolysis of keratin azure are shown in Figure 3.5. The results show the average keratinolytic activity (expressed in Units/ml) as a function of time for each enzyme treatment. The enzymatic hydrolysis of keratin azure, monitored over a 48 hour period showed the digestion of the substrate was slow in the initial first hour of incubation suggesting less accessibility of the substrate to the enzymes.

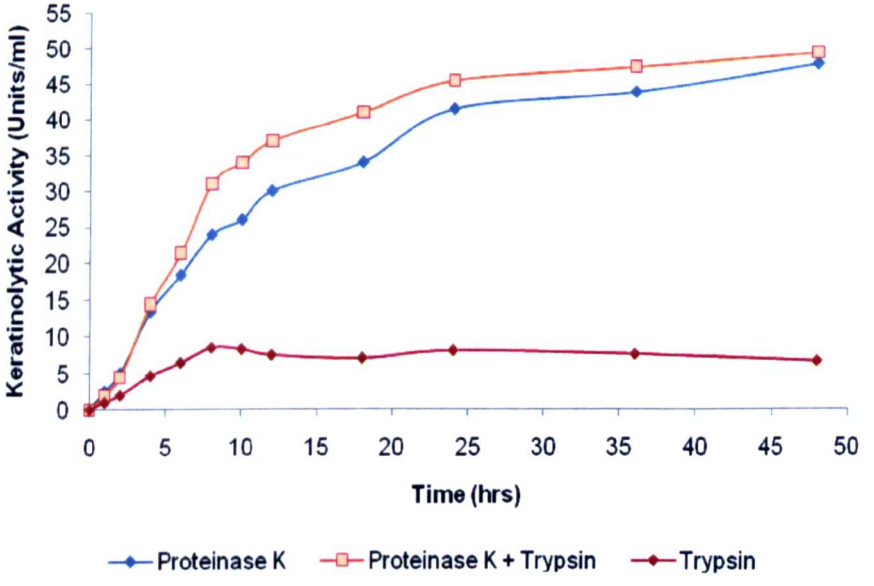

Figure 3.5: Comparative enzymatic hydrolysis of keratin azure with proteinase K, trypsin, and combined proteinase K and trypsin enzymes at pH 8.0 and 40°C for 0 - 48 hours.

Within the first hour of incubation the hydrolysing activity calculated was 2 Units/ml for the enzyme mixture proteinase K and trypsin, 2.5 Units/ml for proteinase K, and 1 Unit/ml for trypsin enzyme. After 6 hours of incubation the activity of the enzyme mixture proteinase K plus trypsin, and proteinase K showed a comparable increase while the trypsin enzyme exhibited a significantly lower activity. The calculated activity was 22 Units/ml for the enzyme mixture proteinase K and trypsin, 19 Units/ml for proteinase K, and 7 Units/ml for trypsin. This represented an increase of about 10 fold for proteinase K and a combined proteinase K and trypsin digestion, respectively. The highest activity calculated for tryptic digestion was 9 Units/ml, attained after a 8 hour incubation period, after which there was no increase in the intensity of soluble dye suggesting no further digestion by the enzyme (Figure 3.5). This may be attributed to the inability of the enzyme to digest the substrate further, and possibly saturation of the enzyme with degradation products.

During the incubation experiments, it was observed that the hydrolytic activity of the combined enzyme mixture of proteinase K plus trypsin was consistently higher than that of proteinase K between 8 hours to 24 hours incubation period. The average activity calculated from the intensity of soluble dye (absorbance) was 31, 34, 37, 41, and 46 Units/ml, for the enzyme mixture of proteinase K plus trypsin for the incubation period ranging from 8, 10, 12, 18, and 24 hours, respectively. Whereas the average activity calculated for proteinase K during the same incubation period were 24, 26, 30, 34, and 42 Units/ml, respectively. The observed differences suggest that as the reaction progressed the hydrolysis of the substrate improved with the combined enzyme mixture, which resulted in a more soluble azure dye being released. It is possible, that as a consequence of progressive attack of the highly crosslinked components of keratin by proteinase K other protein constituents which are less crosslinked were exposed thus becoming vulnerable to tryptic digestion. The results further demonstrate that although trypsin and proteinase K may both belong to the class of serine proteases, they certainly act on different substrates.

Figure 3.6 shows the results of the average absorbance as a function of time. The rate of dye release in all the enzyme assays increased linearly with respect to incubation time for the initial 8 hours of incubation. The initial reaction rates calculated from the slope of the linear part (Figure 3.6) and expressed as a change in absorbance per unit of time showed an increase in the initial velocity of reactions, V_0, for trypsin and proteinase K when the two enzymes were combined and used as a single treatment. The initial reaction rates, V_0, calculated for each enzyme treatment were 3.6×10^{-4}/min for trypsin, 1.03×10^{-3}/min for proteinase K, and 1.44×10^{-3}/min for the combined proteinase K and trypsin enzymes. By comparing the initial reaction rates of proteinase K and that of the combined enzyme

treatment it shows there was a 40% increase in the initial reaction rate with the combined enzyme treatment. This evidence supports the increased activity observed in the treatment involving the combined enzymes (Figure 3.5).

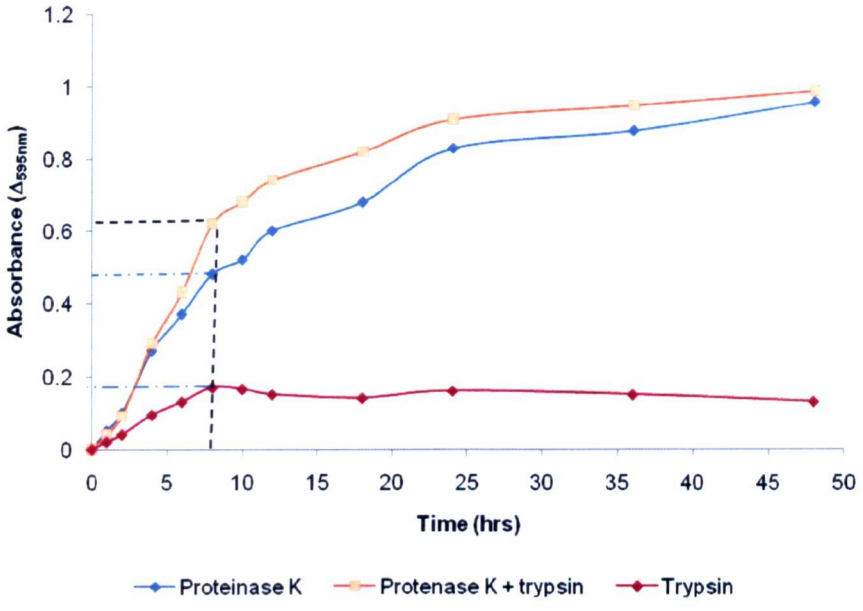

Figure 3.6: Average absorbance as a function of time following incubation of keratin azure with proteinase K, trypsin, and a combined proteinase K and trypsin enzymes at pH 8.0 and 40°C for 0 - 48 hours . The absorbance was measured at 595 nm.

3.5.3 Enzymatic degradation of bovine hair with proteinase K

Proteinase K is a serine protease isolated from fungus of the genus *Tritirachium album*, with the characteristic ability to degrade native proteins including keratin substrates (Ebeling *et al.* 1974; Sweeney and Walker, 1993). It is an endo-peptidase which cleaves peptide bonds preferentially adjacent to the carboxyl group of aliphatic and aromatic amino acids (Kraus *et al.*, 1976). Figure 3.7 represents the profiles of the concentration of soluble hair proteins detected following incubation with proteinase K. The concentration of soluble protein expressed in mg/ml, shows no differences between the samples, brown, black and white bovine hair. The average concentration of solubilised hair protein detected in the first hour of incubation was 0.14 mg/ml, increasing to an average of 0.57 mg/ml in 24 hours. The average concentration of soluble protein detected after 72 hour incubation was 0.61 mg/ml. The initial reaction rate calculated from the slope of the tangent for each substrate expressed as the change in the concentration of soluble protein per unit of time was 1 µg/ml min^{-1} for each of the brown and white hair substrates, and an average of 1.3 µg/ml min^{-1} for the black hair substrates. The results indicate that the substrates were being hydrolysed at

the same rate, and further suggest that the presence of melanin may not have any influence on the rate of digestion.

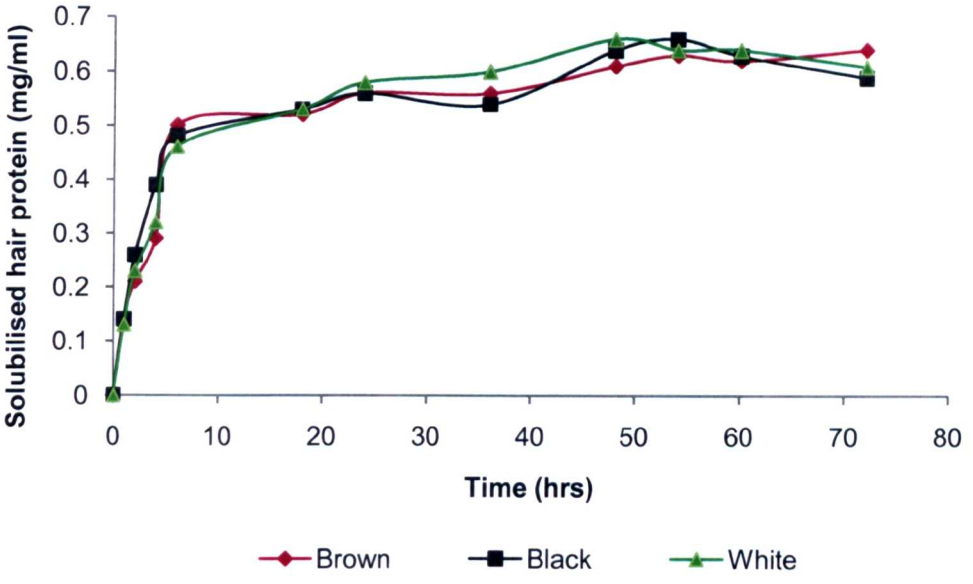

Figure 3.7: The concentration of solubilised hair protein following incubation of brown, black and white bovine hair substrates with proteinase K at pH 8.0 and 40°C for 72 hours.

The molecular characteristics of the soluble products obtained after 72 hours enzymatic incubation with proteinase K are shown on Figure 3.8. Lane 2 is the Sigma low molecular weight protein standard, lane 3 is proteinase K enzyme in sample buffer, lane 4 is the dilution buffer, lanes 5, 6, and 7 represent the electrophoretic profiles of white, black and brown hair proteins. The soluble proteins were resolved at one major molecular mass band estimated at approximately 70kD (Figure 3.8). The results show similarities in the specificity of the enzyme on the substrates, which further suggest that the presence of melanin did not alter the way keratinase (proteinase K) cleaved the polypeptides of bovine hair proteins.

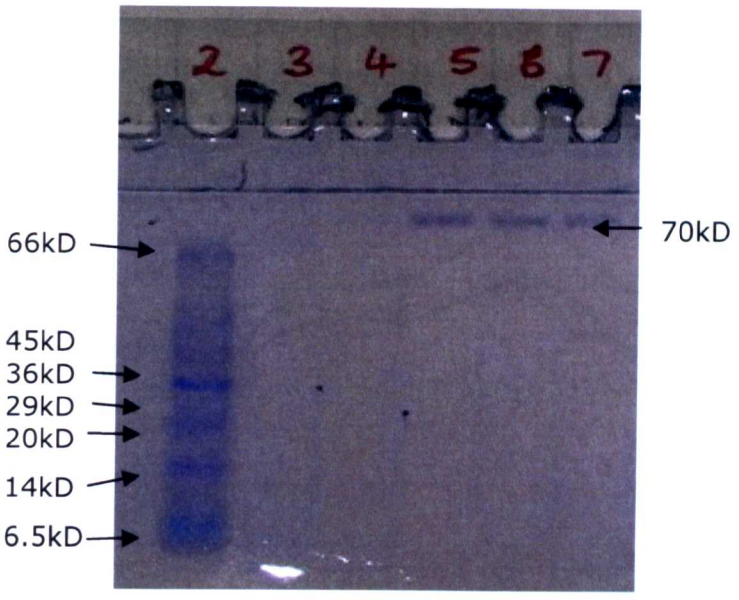

Figure 3.8: SDS-PAGE of bovine hair soluble proteins following incubation with proteinase K for 72 hours. Lane (2): standard, (3): proteinase K in buffer, (4): dilution buffer, (5): white hair, (6): black hair, (7): brown hair

3.5.4 Enzymatic degradation of bovine hair with trypsin enzyme

Trypsin is a serine protease isolated from mammalian pancrease. It is an endo-peptidase with the specificity to catalyse the hydrolysis of peptide bonds adjacent to amino acid residues with basic side chains such as lysine and arginine (Brown and Wold, 1973; Ma *et al.*, 2005; Palmer, 1981). Keratin is known to be resistant to the action of proteolytic enzymes like trypsin, papain and pepsin (Fuchs, 1995; Goddard and Michaelis, 1934). Speakman and Yarwood (1966) also observed that untreated wool was not attacked by trypsin. However, in a previous experiment, Routh and Lewis (1938) observed partial digestion of powdered keratin by trypsin enzyme. Blackburn (1950), further reported that under suitable conditions of temperature and pH keratins were attacked by trypsin and pancreatin. Figure 3.9 shows the results of the concentration of soluble hair proteins detected following incubation with the trypsin enzyme. The degradation of hair was observed to be slow with an average protein concentration of 0.14 μg/ml in the first hour, 22 μg/ml in 24 hours, and the highest concentration of soluble protein of 25 μg/ml being detected after 48 hours incubation. After 48 hours no increase in the concentration of protein solubilised was detected, indicating no further protein degradation was taking place.The initial reaction rates calculated from the linear part of the curve for each of the substrates and expressed as the change in the concentration of soluble protein per unit of time, were 0.02 μg/ml min^{-1} for brown hair substrates, 0.05 μg/ml min^{-1} and 0.04 μg/ml min^{-1} for the black and white hair

substrates, respectively. These values are relatively low in comparison to the values (1 μg/ml min^{-1} and 1.3 μg/ml min^{-1}) obtained for soluble hair protein with proteinase K digestion.

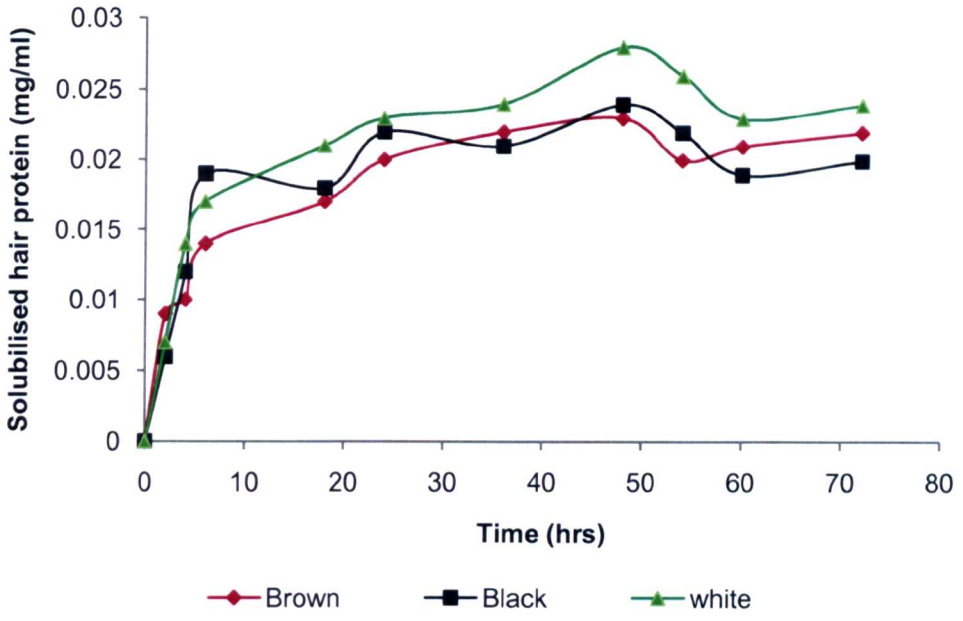

Figure 3.9: The concentration of soluble hair proteins following incubation of brown, black and white bovine hair substrates with the trypsin enzyme at pH 8.0 and 40°C for 72 hours.

The relative slow digestion of hair by the trypsin enzyme may be attributed to the complex structure of hair, the tightly packed structure of keratin and the variability of its protein constituents. The highly crosslinked (keratinised) hair components are more resistant to degradation by proteolytic enzymes (Fraser *et al.*, 1972; Jones and Rivett, 1997; Stoves, 1948). Keratins such as hair, wool, and feathers contain a mixture of proteins with a sizeable fraction of non-keratinous proteins, which are less crosslinked and easily degraded (Bauman, 1979; Bienkeiwicz, 1983). The medullary proteins are non-keratinous and, like the proteins of the inner root sheath of hair, can be degraded by proteolytic enzymes (Bauman, 1979; Dawber, 1996; Fraser *et al.*, 1972). The cytoplasmic and intercellular materials distributed as a network in the keratinous material and embedded within the cortex are vulnerable to proteolytic degradation (Bauman, 1979; Jones and Rivett, 1997; Wolfram, 2003). Steinert *et al.*, (1971) successfully demonstrated the ability of the trypsin enzyme to digest the proteins of the inner root sheath of hair. The soluble hair proteins detected during incubation with trypsin enzyme, therefore, may be from the non-keratinous components, such as medulla, the intercellular and cytoplasmic materials.

3.5.5 Comparative enzymatic degradation of bovine hair with proteinase K, and combined proteinase K plus trypsin enzymes

The comparative results obtained from the digestion of bovine hair with proteinase K, and combined proteinase K plus trypsin enzymes are shown in Figure 3.10.

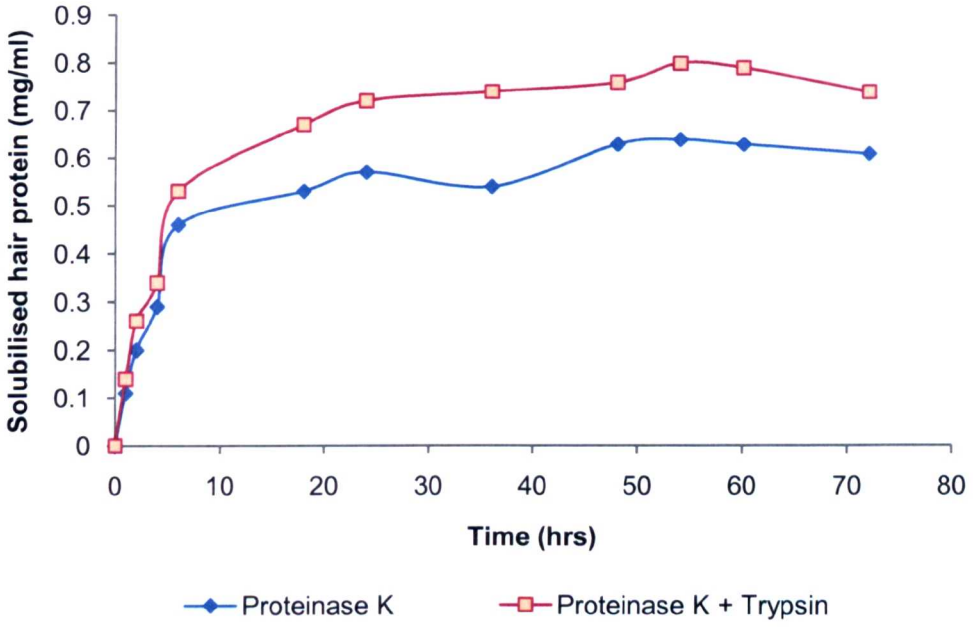

Figure 3.10: Comparative concentration of solubilised hair proteins following enzymatic incubation of bovine hair with proteinase K, and combined proteinase K and trypsin enzymes at pH 8.0 and 40°C for 0 - 72 hours.

The results are expressed as average concentration of protein detected from each enzyme treatment (*i.e.* proteinase K, and combination of proteinase K plus trypsin, respectively). It was observed that the concentration of detectable soluble protein gradually but steadily increased with the combined enzyme, proteinase K plus trypsin treatment, during the incubation period. The concentration of hair protein solubilised by each enzyme treatment at different incubation period is given in Table 3.1. For the treatment with combined proteinase K and trypsin enzymes, the average concentration of soluble protein detected was 0.11 mg/ml in the first hour of incubation, 0.67 mg/ml in 18 hours, 0.72 mg/ml in 24 hours, 0.76 in 48 hours, and 0.74 mg/ml after 72 hours. In the case of proteinase K the average concentration of protein detected during the same period was 0.14 mg/ml in the first hour, 0.53 mg/ml in18 hours, 0.57 mg/ml in 24 hours, 0.63 mg/ml in 48 hours, and 0.61 mg/ml in 72 hours. The initial reaction rates calculated from the slope of the linear curve for each of the enzyme treatments were 1 μg/ml min^{-1} for proteinase K, and 1.3 μg/ml min^{-1} in case of combined proteinase K plus trypsin enzymes.

Table 3.1: Concentration of hair protein solubilised following incubation with proteinase K, and proteinase K plus trypsin enzymes for 0 – 72 hours. (V_0) is the initial reaction rates for each treatment.

Time (hrs)	Proteinase K		Proteinase K plus trypsin	
	Protein (mg/ml)	(V_0) = 1 µg/ml min^{-1}	Protein (mg/ml)	(V_0) = 1.3 µg/ml min^{-1}
1	0.14		0.11	
18	0.53		0.67	
24	0.57		0.72	
48	0.63		0.76	
72	0.61		0.74	

Although the initial reaction rates (V_0) for proteinase K treatment (1 µg/ml min^{-1}), and that of the combined proteinase K plus trypsin enzymes (1.3 µg/ml min^{-1}) were not siginificantly different the apparent increase in concentration of soluble proteins suggests more hair protein was being degraded by the combined enzyme treatment than by the single treatments involving protenase K, and trypsin, respectively. This may be explained by the fact that since hair is a composite tissue, with variable protein constituents and variable degrees of crosslinking of its structural components, the two enzymes may have been acting in co-operation (synergistically), but selectively on the different substrates of the hair components. The structural breakdown of hair is influenced by its structural components, the cuticle, cortex and medulla, and their constituent composition (*i.e.* degree of crosslinks) (Addy, *et al.*, 2001; Baumann, 1979; El Baba *et al.*, 2000). The structural protection of the components is provided by the cuticle (Cubarsi *et al*, 2006; Stoves, 1948). Therefore, it may be suggested that proteinase K (keratinolytic enzyme) targets the cuticle and other highly keratinised components, thus opening up the structure that facilitates accessibility to proteolytic digestion of the non-keratinised components by the trypsin enzyme, yielding soluble proteins.

The above explained combined enzymatic action on the different components of hair, where proteinase K breaks down the keratinised hair components while trypsin targets the non-keratinised components replicates the effects of the chemical pretreatments of the combined action of potassium thioglycolate and sodium hydroxide (NaOHKTG pretreatment, Chapter 2, Section 2.4.3.3). In that particular pretreatment (NaOKTG), the structural breakdown of hair was enhanced with potassium thioglycolate (KTG) as a reducing agent breaking down the disulfide bonds, and an alkali (NaOH) as a hydrolysing agent breaking down the non-disulfide crosslinked proteins.

3.5.6 The effect of potassium thioglycolate and sodium hydroxide pretreatment on enzymatic degradation of bovine hair

Proteolytic digestibility of keratin is limited by the disulfide crosslinking of the polypeptide chains of the amino acid cystine, which also contributes to the high mechanical stability (Fraser *et al.*, 1972; Jones *et al.*, 1997). Alkali pretreatment has been commonly used to improve the enzymatic digestibility of keratinous substrates. The general effect of the chemical pretreatments (Papadopoulos *et al*, 1985; Steiner, 1983) has been described to cause a disruption of the structural integrity of the highly resistant substrates in order to improve accessibility to the degrading enzymes to complete the breakdown of the substrate. In this study, the results of the effects of pretreatment on enzymatic digestion of hair are shown on Figure 3.11. These results represent the average concentration of soluble protein detected as a function of incubation time and are compared with the results of enzymatic hydrolysis of non-pretreated hair (intact hair). The initial reaction rates (V_0) calculated from the slope of the curve for each of the treatments (enzymatic hydrolysis of NaOHKTG pretreated hair, and the combined enzyme hydrolysis of intact hair) was 1.3 μg/ml $m.^{-1}$ However, the concentration of soluble protein increased four times, from an average of 0.11 mg/ml for non pretreated hair substrate to 0.44 mg/ml for the pretreated hair substrate during the first hour of incubation, indicating chemical pretreatment enhanced enzymatic solubilisation of hair protein further.

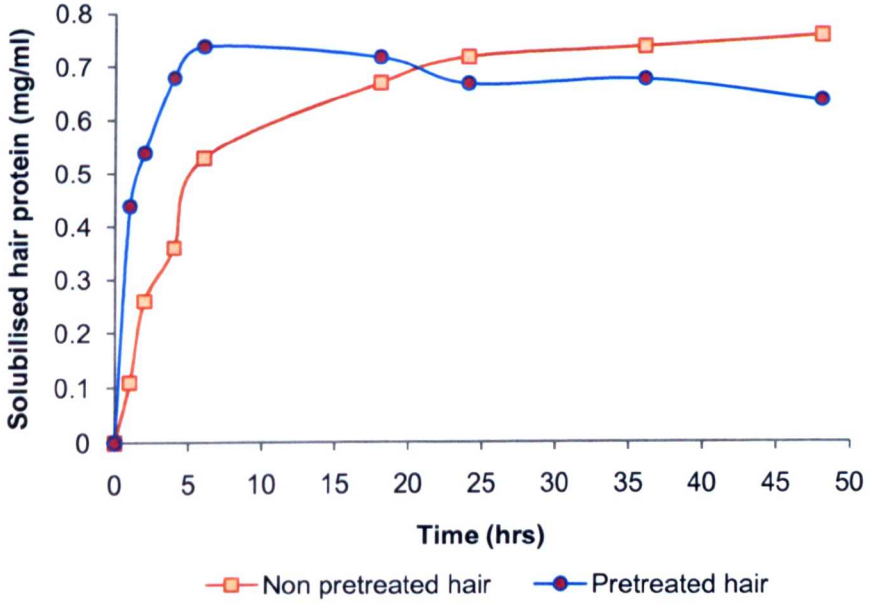

Figure 3.11: Comparative concentration of solubilised hair protein following enzymatic incubation of chemically pretreated and intact bovine hair with combined proteinase K plus trypsin enzymes, at pH 8.0 and 40°C for 48 hours.

During the progressive incubation of pretreated hair with the combined enzyme mixture, the average concentration of soluble protein released was 0.74 mg/ml after 6 hours of incubation, while the concentration of soluble protein detected during the same period with non pretreated hair substrates was 0.53 mg/ml. It was noted that after 6 hours there was no further increase in the concentration of soluble proteins and the digestion curve for pretreated hair levelled off suggesting the enzymatic digestion might have slowed down. Besides slowing down of the enzymatic hydrolysis it can be seen from the digestion curve of the pretreated hair that after levelling off the average concentration of soluble protein steadily declined, reaching an average of 0.64 mg/ml after 48 hours incubation. This may be attributed to competitive inhibition of the enzymes by the products of hydrolysis.

3.5.7 Physical structure changes (SEM analysis)

3.5.7.1 Effect of enzymatic degradation of bovine hair with proteinase K

Figures 3.12 and 3.13 are microscopy (SEM) images of intact hair used in this experiment as references (control samples). Changes in physical structure following enzymatic incubation of hair with proteinase K monitored using SEM are represented in Figures 3.14 – 3.17.

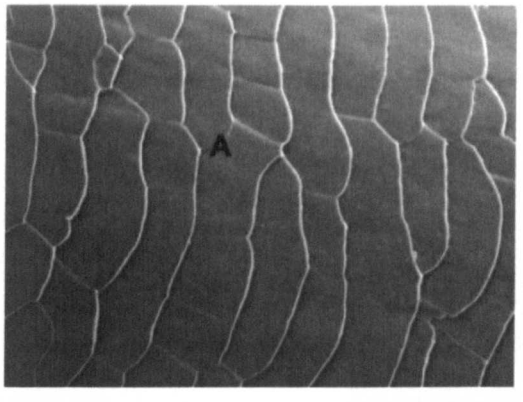

Figure 3.12: SEM image of intact hair shaft: (A) cuticle (mag. x1.0K, 5.0kV).

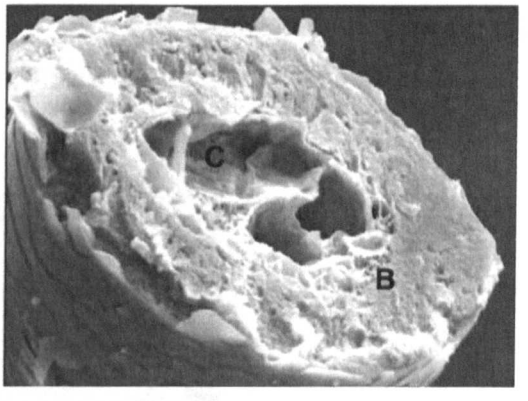

Figure 3.13: SEM image of a cross-section of an intact hair shaft: (B) cortex, and (C) medulla (mag. x1.0K, 5.0kV).

Figure 3.14 shows the initial stages of hair degradation by the enzyme, proteinase K, to be on the cuticle. The early stages of degradation was characterised by the erosion of the cuticle surface, particularly at the base between the cuticle cells (Figure 3.14). An attack on this region was observed to cause the flaking of the individual cuticle cells which suggests that the cementing layer, the cell membrane complex (CMC), was the target of enzymatic attack.

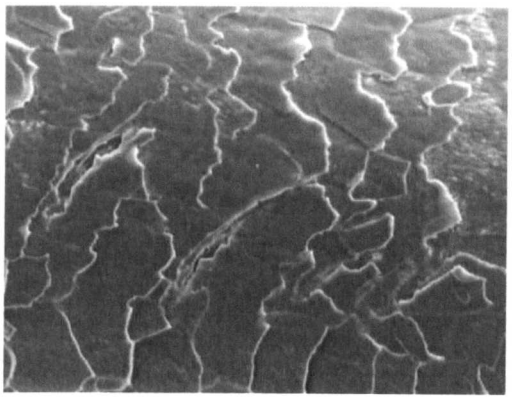

Figure 3.14: SEM image illustrating the damage to the hair shaft following incubation with proteinase K after 1 hour (mag. x1.0K, 5.0kV).

The cell membrane complex is believed to be composed of less crosslinked proteins and glycoproteins (Allen *et al.*, 1991; Swift and Smith, 2001), thus it is more vulnerable to a keratinase and proteolytic attack. Progressive degradation of the cementing layer and the inner region of the cuticle is illustrated in Figure 3.15. From the schematic diagram of cross-section of the hair cuticle (Figure 1.8, Chapter 1) the inner region which is vulnerable to proteolytic degradation is the low-sulfur containing, endocuticle (Dawber, 1996; Jones, 2001; Swift and Smith, 2001). From the SEM image (Figure 3.15) there is evidence to suggest that enzymatic degradation of the cementing intercellular layer was followed by enzymatic attack on the cuticle from the underlying endocuticle region, leading to the cuticle flaking off.

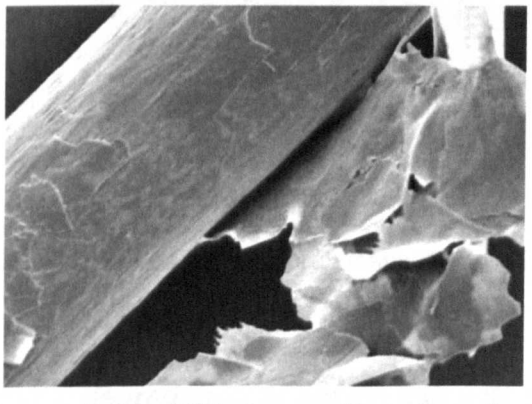

Figure 3.15: SEM image illustrating the damage of the intercellular layer and the cuticle following incubation with proteinase K after 48 hours (mag. x1.0K, 5.0kV).

The damage to the structure of hair by proteinase K was observed to progress with incubation time. Figures 3.16 and 3.17 are illustrations of the extent of structural damage after 72 hours of incubation. Figure 3.16 show the smooth surface of the hair shaft following degradation of the intercellular material and the cuticle.

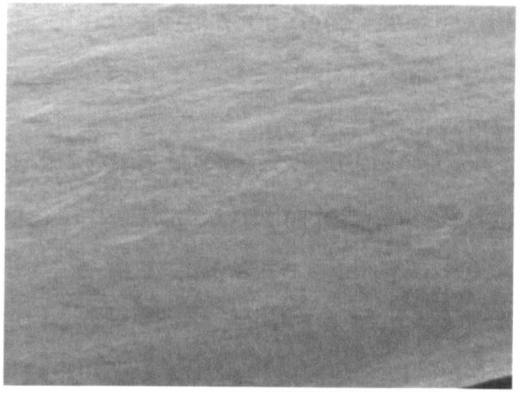

Figure 3.16: SEM image illustrating degraded hair shaft following incubation with proteinase K after 72 hours (mag. x1.0K, 5.0kV).

The undegraded structure is believed to be the highly keratinised macrofibrils of the cortex and the cementing material that holds the fibrils together. Figure 3.17 shows the dissolved medulla and the damaged cortex. The dissolution of the medulla demonstrates that in addition to its keratin degradation potential proteinase K was capable of degrading some of the non-keratinised components.

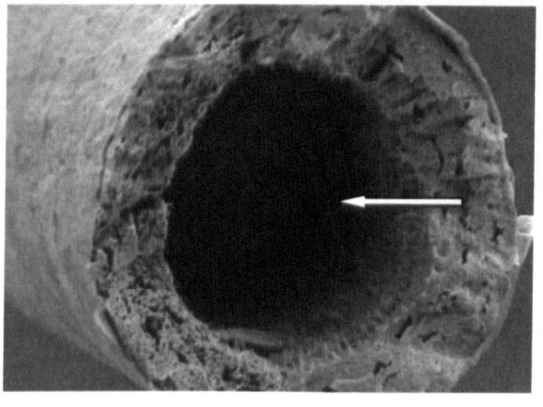

Figure 3.17: SEM image illustrating the damage to the medulla and cortex (see arrow) following incubation with proteinase K after 72 hours (mag. x1.0K, 5.0kV).

3.5.7.2 Effect of enzymatic degradation of bovine hair with trypsin

Keratins have been described as proteins of epidermal and skeletal tissues which are insoluble in the usual protein solvents, as they are high in cystine content, and resistant to proteolytic enzymes such as trypsin and pepsin (Fraser *et al.*, 1972; Goddard and Michaelis, 1934). However, from the chemical point of view it is also known that keratins such as wool, hair, horn, nails and feathers are composed of a mixture of proteins of different composition

which include small amounts of non-keratinous (less crosslinked) proteins, soluble proteins as well as some non-proteinaceous material (Bauman, 1979; Mercer, 1953).

Physical structural changes, following tryptic enzyme digestion of hair, are represented by Figures 3.18 – 3.20. The relative resistance of the keratinised hair components to proteolytic degradation is evidenced by the fact that even after 48 hours of incubation with trypsin enzyme the cuticle remained largely intact, Figure 3.18. Figure 3.19 shows a deformed hair shaft, with a slightly collapsed structure after 72 hours of tryptic digestion, which suggests, structural damage had occurred. The collapsed nature of the hair shaft provides evidence which suggests that the medulla may have been dissolved.

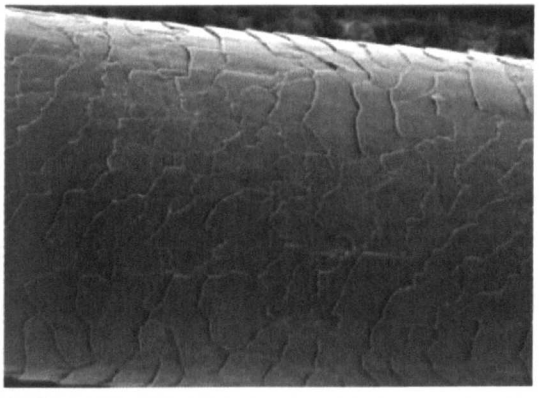

Figure 3.18: SEM image illustrating the structure of the hair shaft following incubation with trypsin enzyme after 48 hours (mag. x1.0K, 5.0kV).

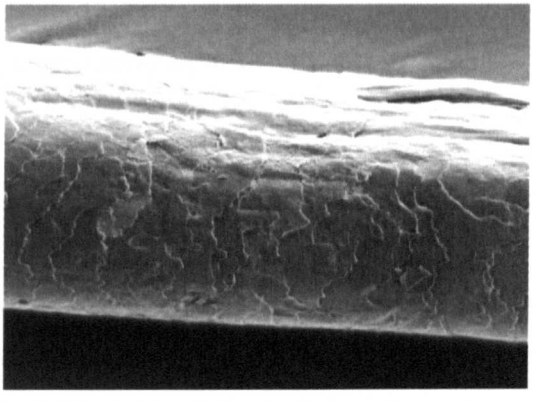

Figure 3.19: SEM image illustrating damage to the hair shaft following incubation with trypsin enzyme after 72 hours (mag. x1.0K, 5.0kV).

Figure 3.20 shows the cross-section view of the hair shaft, further illustrating the effect of tryptic digestion on the medulla after 72 hours of incubation. The medulla appears dissolved and the cortex partially degraded (see arrow), while the cuticle (A) remains intact. The medullary cells are composed mainly of the protein citrulline, which like the inner root sheath, can be degraded by proteolytic enzymes (Blackburn, 1948; Fraser *et al.*, 1972; Orwin, 1979; Rogers, 1962). These results demonstrate that whereas trypsin was able to degrade the non-keratinised protein fractions it was not enough to cause significant damage to the physical structure of hair and that the enzyme (trypsin) had little effect on the highly keratinised components of hair.

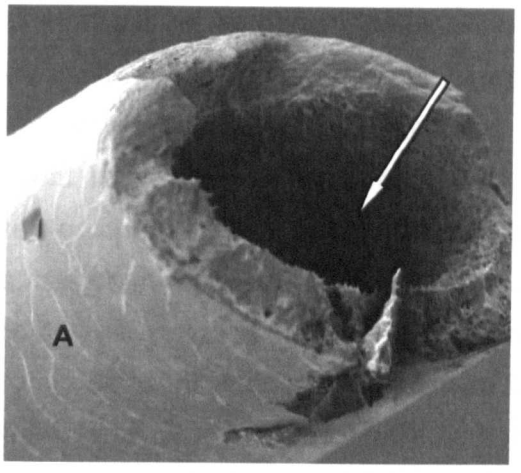

Figure 3.20: SEM image of hair shaft illustrating damage to the medulla (see arrow) following incubation with trypsin enzyme after 72hours (mag. x1.0K, 5.0kV). (A) the cuticle.

3.5.7.3 Effect of degradation of bovine hair with combined proteinase K and trypsin enzymes

Figures 3.21 and 3.22 are representative microscopy (SEM) images of changes in morphology of bovine hair following incubation with combined proteinase K and trypsin enzymes, for 48 and 72 hours, respectively. The microscopy images show the effectiveness of the two enzymes combined in breaking down the hair structure, compared to when the enzymes were used as individual treatments (Figures 3.17 & 3.20). The difference is seen in the distinct and enhanced fibrillation of the hair cortex (Figures 3.21 & 3.22, see arrow), when the two enzymes (proteinase K and trypsin) were used together.

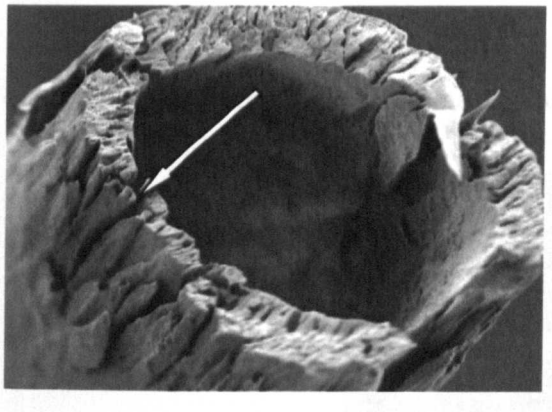

Figure 3.21: SEM image illustrating the damage to the hair shaft following incubation with proteinase K and trypsin enzymes after 48 hours (mag. x1.0K, 5.0kV).

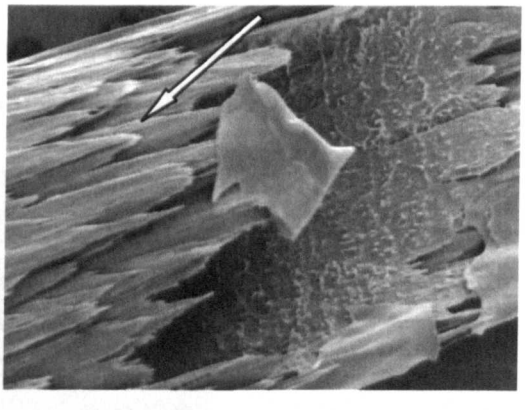

Figure 3.22: SEM image illustrating fibrillated hair cortex (see arrow) following incubation with proteinase K and trypsin enzymes after 72 hours (mag. x1.0K, 5.0kV).

The fibrillation (separation of the fibres) process was observed to be progressive with incubation time. The fibrillation of the cortex demonstrates enhanced structural breakdown as a result of the cooperative action of the two enzymes, in which the selective degradation of the non-keratinous proteins, intercellular materials and keratin proteins occurs simultaneously but at different rates. The less crosslinked proteins were digested first leaving the highly keratinised components. The cooperative action (usually designated as synergy) between enzymes, has been reported mainly between various cellulose degrading enzymes (Hoshino *et al.*, 1997). The observed morphological changes support the increased concentration of soluble protein detected during the incubation experiments (Figure 3.10). The observation was that the overall degree of enzymatic hydrolysis of hair improved with the enzyme mixture. The significance of this is that the use of mixed enzymes offers the potential of an efficient and improved degradation of hair, which may be compatible with the composting process.

3.5.7.4 Effect of chemical pretreatment on the enzymatic degradation of bovine hair

Most proteins in their native state are known to be attacked slowly, if at all, by proteolytic enzymes. Usually, denaturation treatments causing partial or total disruption of the protein conformation lead to increased susceptibility to proteolysis (Stevens and Sauberlich, 1970). De Graaf (2000) claimed that denaturation is a prerequisite for processing of proteins into a product and for obtaining the desired properties of the final product. In the case of recalcitrant animal proteins, such as keratin, pretreatments are aimed at disrupting the cystine disulfide bonds to improve enzymatic digestibility (Papadopoulos, 1985; Steiner *et al.*, 1983; Wang and Parsons, 1997).

Figure 3.23 is a representative SEM image of hair previously chemically pretreated with combined potassium thioglycolate and sodium hydroxide (NaOHKTG) for 1 hour.

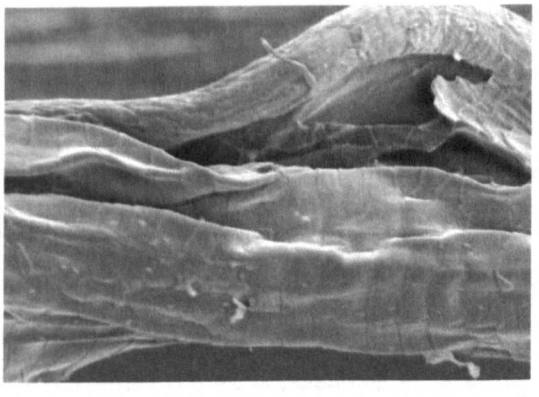

Figure 3.23: SEM image of hair chemically pretreated with potassium thioglycolate and sodium hydroxide for 1 hour (mag. x1.0K, 5.0kV).

Figures 3.24 and 3.25 represent the physical characteristics of the pretreated hair samples following enzymatic digestion with a mixture of proteinase K and trypsin enzymes. The results show that pretreatment enhanced the enzymatic disintegration of hair compared to enzymatic digestion of intact hair samples (Sections 3.5.7.1 – 3.5.7.3). Figure 3.24 shows the disintegrated hair structure after 10 hours of incubation with the enzyme mixture. There was a loss of the structural integrity, characterised by the sheet-like cuticle debris. Although prolonged incubation for 48 hours saw further disintegration of the structure, the cuticle was not completely degraded (Figure 3.25).

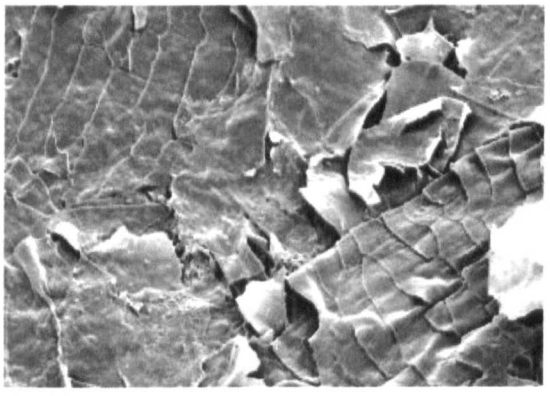

Figure 3.24: SEM image illustrating damage to the chemically pretreated hair following incubation with proteinase K and trypsin enzymes after 10 hours (mag. x1.0K, 5.0kV).

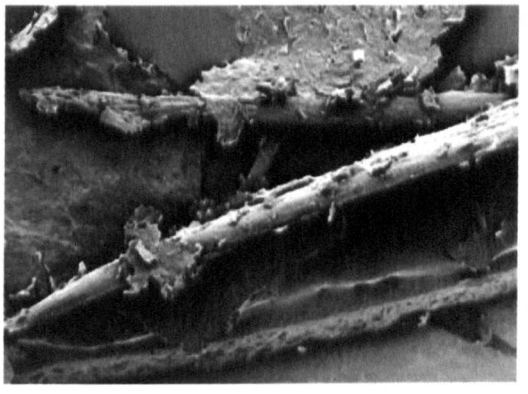

Figure 3.25: SEM image illustrating damage to the chemically pretreated hair following incubation with proteinase K and trypsin enzymes after 48 hours (mag. x1.0K, 5.0kV).

Although chemical pretreatment involving the use of potassium thioglycolate and sodium hydroxide greatly enhanced digestibility and disintegration of hair, from a technical point of view, the overall compatibility with the composting process and economic cost may be a major challenge. The alternative approach to improve the degradation of hair during composting, may be, the use of an enzyme mixture. As already observed in this study (Sections 3.5.5 & 3.5.7.3), an efficient enzymatic process requires the incorporation of different enzymes to effectively breakdown the multi-complex hair structure. The concept of using a consortium of enzymes to degrade biopolymers is not new, and has been used mainly in the bio-processing of cellulose (Cao and Tan, 2005; Rabinovich *et al.*, 2002; Zhang and Lynd, 2004). Cellulose is a naturally occurring polymer which resembles hair due to its composite structure. Micro-organisms are commonly used as source materials for a variety of commercial enzymes (Godfrey and West, 1996; Rao *et al.*, 1998). In nature, keratins can be hydrolysed by some micro-organisms which synthesise keratinolytic enzymes (Onifade *et al.*, 1998). The availability of micro-organisms with the potential to degrade hair keratin is explored in the next chapter (Chapter 4), as a possible alternative to the use of commercial enzymes.

3.6 Summary of the chapter

- The commercial enzyme, proteinase K, used as source of keratinase, was effective in breaking down hair, with an average of 0.14 – 0.61 mg/ml of protein solubilised during the incubation period of 1 – 72 hours, and an initial reaction rate (V_0) of 1 μg/ml min^{-1}. However, a complete breakdown of hair was not achieved during the experimental period. SDS-PAGE analysis of the products of hydrolysis produced one major molecular weight band in the range of 70kD for each of the hair substrates (brown, black and white). The enzyme selectively attacked the hair components, preferentially initiating through the cell membrane complex, the endocuticle, medulla, and the less crosslinked fibrils of the cortex.

- The commercial enzyme, trypsin, used as a protease in the experiment was inefficient in digesting hair. However, under the experimental conditions, the enzyme was able to hydrolyse hair proteins believed to be the non-keratinous and soluble protein constituents as demonstrated by its ability to dissolve the medulla while the cuticle and cortex remained intact.

- Enzymatic degradation of hair using a combined enzyme formulation containing proteinase K, acting as a keratinase, and trypsin as a protease, improved the hydrolysis and structural breakdown of hair. This was demonstrated by the increasing average concentration of protein solubilised, and microscopy (SEM) evidence of fibrillation of the hair cortex. The average concentration of protein solubilised ranged from 0.11 – 0.74 mg/ml during the incubation period of 1 – 72 hours, with an initial reaction rate (V_0) of 1.3 μg/ml min^{-1}. Fibrillation of the cortex was evidence of improved disintegration of the structure, which further demonstrated the potential of the designed enzymatic process to replace the chemical process of dissolving hair.

- Pretreatment with potassium thioglycolate and sodium hydroxide enhanced enzymatic breakdown of hair. The average concentration of protein solubilised during the experimental incubation of 1 – 48 hours ranged from 0.44 – 0.64 mg/ml. The microscopy evidence revealed that although other hair structural components were completely disintegrated, the cuticle was not dissolved.

The next Chapter (Chapter 4) of this thesis will focus on identifying the micro-organisms capable of effectively breaking down hair as a more cost-effective source of enzymes, and to build on the concept of synergy between enzymes.

CHAPTER 4

MICROBIAL DEGRADATION OF BOVINE HAIR

CHAPTER 4

4. INTRODUCTION

4.1 Microbial keratinases

Microbial enzymes are often considered more useful than enzymes from sources such as plants, or animals, due to their diverse catalytic activities. In addition, since the micro-organisms can be made to propagate rapidly on inexpensive media, microbial enzymes can be obtained in high yields, are easy to control, and guarantee regular and reliable supply. They are also more stable across a broad pH range and temperatures (Hasan *et al.*, 2006). In the previous chapter (Chapter 3), the decomposition of hair structure was observed to be enhanced during an enzymatic hydrolysis process, in which two types of commercial enzymes acted cooperatively to breakdown the keratinous and non-keratinous constituents of hair. Micro-organisms which synthesise keratinases, that are capable of degrading keratins, are reportedly produced by fungi, and several bacteria such as *Streptomyces* and *Bacillus* species, respectively. The presence and distribution of these bacteria in the natural habitat is characterised by their ability to metabolise the recalcitrant keratinous substrates (Brandelli, 2008; Gupta and Ramnani, 2006; Onifade *et al.*, 1998; Weitzman and Summerbell, 1995). Microbial keratinases mainly belong to the extracellular serine-type proteases (Brandelli, 2008; Horikoshi, 1996; Rao *et al.*, 1998).

The aim of this chapter is to identify a cheaper source of a biological agent capable of effectively breaking down hair. This chapter will also discuss the mechanism of microbial degradation of hair and the enzyme-enzyme synergistic action as a possible mechanism to improve the efficiency of hair degradation, as observed in Chapter 3. This section of research comes as the precursor to the composting chapter (discussed in Chapter 5), and will serve to facilitate the development of parameters for the composting process. The primary objectives in addressing the aim are:

- To demonstrate the ability of micro-organisms to degrade hair,
- To determine the mechanism by which the micro-organism attacks hair with regard to the sequence of breakdown of different structural components,

- To identify, isolate and characterise the micro-organisms responsible for hair degradation
- To optimise the parameters (growth conditions, pH and temperature) for the microbial degradation of hair.
- To analyse the physicochemical changes as a result of microbial degradation.

4.2 Materials and methods

4.2.1 Sample preparation

Bovine hair samples (brown, black and white) were prepared as previously described (Chapter 2, Section 2.6.1). Fresh soil samples, collected randomly from a location in the field next to the tannery of the British School of Leather Technology, were sieved through a 2 mm sieve and used as a source of micro-organisms.

4.2.2 Laboratory studies on microbial biodegradation of bovine hair

The purpose of this experiment was to demonstrate the presence of micro-organisms with the potential to degrade hair and to determine the mechanism of degradation with respect to the decomposition of its structural components. Laboratory studies, therefore, provided a method by which the decomposition could be studied under controlled, constant environmental conditions.

Intact bovine hair samples (0.2 g) were incubated with soil (30 g) (average moisture content, 53 ± 4%), on a sterile Petri dish in a 40°C oven. The incubation was carried out aerobically, with regular inspection at 0, 5, 10, 15, 20, 25 and 30 days. The moisture supply was maintained by keeping a tray of water in the oven during the incubation period. Each experiment was carried out in duplicate. A control sample containing hair without soil, was placed alongside the test samples. The substrates (hair) were sampled at the end of each incubation period, washed with deionised water, rinsed in 50% (v/v) alcohol and prepared for electron microscopy (SEM) as described in Chapter 2, Section 2.2.2.1.1.

4.2.3 Culture media, growth conditions and micro-organism

The alkaline culture media, pH 11, was prepared according to Horikoshi (1996). The culture media containing KH_2PO_4 (0.5 g), Na_2CO_3 (5 g), $Mg_2SO_4.7H_2O$ (0.01 g), (Fisher Scientific, UK), and 0.1 g of bovine hair (as the only source of carbon and nitrogen) was prepared in a 500 ml Erlenmeyer flask and sterilised by autoclaving at 120°C for 15 minutes. The soil sample (0.2 g) previously incubated with bovine hair after 7 days (Section 4.2.2) was added into the alkaline culture media as a potential source of keratinolytic micro-organisms. The incubation was carried out under aerobic conditions in an orbital rotary incubator at temperatures ranging from 40 –

50°C for 48 – 72 hours. During the growth period, microbial degradation of hair was assessed by sampling hair and examining the physical structure changes using the scanning electron microscope (SEM) according to Section 2.2.2.1.1. In addition, aliquots (2 ml) of the culture broth were periodically sampled and incubated with keratin azure (a keratinase substrate) for 24 hours to observe dye release as evidence of keratin degradation. The culture broth (1 ml) from samples that showed degradation of keratin was used to further propagate the bacterium in fresh alkaline culture media (described above) with hair as the only source of carbon and nitrogen. The incubation was carried out for 48 – 72 hours as described above.

4.2.4 Identification of the keratinolytic micro-organism

The micro-organism was grown in nutrient agar plates containing selective media for *Bacillus* (Oxoid, UK), at 30°C for 24 hours, (Appendix G). Microbial staining was carried out by the standard gram-staining procedure (Collins *et al.*, 1995), and the bacterial species identified according to morphological characteristics as described by Gibson and Ruth (1974).

4.2.5 Optimisation of parameters for keratinase production

The parameters studied included incubation temperature of 40 – 50°C, and an incubation period of 24 – 72 hours.

4.2.6 Extraction and partial purification of keratinolytic enzymes

The extracellular microbial enzyme was recovered from the culture media by centrifugation at 10000 r.p.m (Jouan GR20.22), and 4°C for 20 minutes. The supernatant was stored at 4°C and used as a crude enzyme preparation. The microbial crude enzyme extract (1 ml) was diluted with 2 ml of deionised water. The diluted solution was used for the determination of proteolytic and keratinolytic activities as described in Section 4.2.7. The protein concentration in the solution was determined according to the method by Lowry *et al.* (1951) (Section 2.2.2.6.3).

4.2.7 Microbial enzyme assays

4.2.7.1 Assay for keratinolytic activity

Keratinolytic activity and optimum temperature of activity for the isolated crude enzyme was assayed with Keratin azure as the substrate as described in Section 3.4.3.2. Briefly, Keratin azure (10 mg) was incubated at 37°C with the isolated crude enzyme solution (2 ml) in a 50 mM Tris-HCl buffer, pH 7.5, containing 20 mM $CaCl_2$. The incubation was carried out in an orbital shaker for 1 hour. The solubilised substrate was separated by centrifugation (Jouan GR20.22) at 5000 r.p.m at 4°C for 10 minutes. One unit of enzyme activity was defined as the amount of enzyme that catalysed the release of the dye to cause an increase in absorbance of 0.01 at 595 nm after 1 hour under the standard assay conditions (Wainwright, 1982).

4.2.7.2 Assay for proteolytic activity

The protease activity assay using Hide Powder-Remazol Brilliant Blue (10 mg) as the substrate was performed as described in Section 4.2.7.1. Raw bovine skin was also used to further confirm the ability of the enzyme to hydrolyse non-keratinous proteins.

4.2.7.3 Proteolytic inhibitor assays

To determine and characterise the type of microbial protease, the isolated crude enzyme assay solution was treated with 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), a serine protease inhibitor, as described in Section 3.4.4.2, and 50 mM ethylenediaminetetraacetic acid (E.D.T.A.), a metalloprotease inhibitor (Izuka *et al.*, 1993). The incubation was carried out at 50°C for 24 hours.

4.2.7.4 pH and temperature of optimum activity

The pH of optimum activity was studied in 0.2 M Tris-HCl buffer range of pH 7.0, 8.0, 9.0 and 10 for 24 hours. The optimum temperature for keratinase activity was studied by incubating the isolated crude enzyme in 0.2 M Tris-HCl buffer, pH 9.0, with Keratin azure substrate (10 mg). The incubation was carried out in the temperature range of 40 – 60°C under constant agitation

at 100 r.p.m in an orbital shaker for 24 hours. The absorbance was measured at 595 nm on a UV 2501PC spectrophotometer (Shimadzu, Japan).

4.2.8 Hydrolysis of keratin and non-keratin substrates

It should be noted that 0.6 ml of the diluted crude enzyme isolate was the optimised working volume found sufficient to allow progressive monitoring of the enzymatic activities. The protein concentration in the isolated microbial crude enzyme was 15 mg/ml. The protein in the working volume (0.6 ml, of the crude enzyme) was 9 mg.

4.2.8.1 Hydrolysis of bovine hair substrates

The potential of the isolated crude enzyme to degrade the hair substrate was demonstrated by incubating brown, black, and white bovine hair (10 mg) with the isolated crude enzyme solution (0.6 ml), in 0.2 M Tris-HCl buffer, pH 9.0. The total reaction volume was 2 ml, with a final protein concentration of 6.4 mg/ml. The incubation was carried out in an orbital incubator (SI 50, Stuart Scientific, UK), at 50°C with constant agitation at 100 r.p.m for a period ranging 0 – 72 hours. All experiments were carried out in duplicate. Control samples contained the substrate, the crude enzyme in buffer solution plus E.D.T.A, (50 mM). The unsolubilised substrate was separated by centrifugation at 5000 r.p.m at 4°C for 10 minutes. The concentration of protein solubilised was determined in the supernatant by the method of Lowry *et al.* (1951) (Section 2.2.2.6.3).

4.2.8.2 Hydrolysis of non-keratin substrates

To demonstrate the potential of the isolated crude enzyme to degrade non-keratinous protein, fleshed and unhaired solid skin samples (0.1 g) were incubated with the isolated crude enzyme (0.6 ml), in 0.2 M Tris-HCl buffer, pH 9.0, as described above (Section 4.2.8.1).

4.2.8.3 Effect of immunisation on enzymatic hydrolysis of hair substrates

It was mentioned in Chapter 1 that the basis of hair recovery is based on the principles of hair protection against dissolution by a reducing agent. Thus, hair recovered from the tannery during the industrial hair-saving unhairing process is usually exposed to moderate concentrations of lime (calcium hydroxide) to change its chemical characteristics, so that it becomes immune to

destruction by sodium sulfide and other reducing agents. The objective of this experiment was to determine the effects of hair immunisation with respect to the potential degradation by the isolated crude enzyme.

Bovine hair (0.5 g) was added to 50 ml deionised water containing 0.25 g (3.4 mM) of calcium hydroxide in an orbital incubator under constant agitation at 100 r.p.m for 18 hours at 25°C simulating standard tannery process of hair immunisation. The immunised hair was recovered by filtration, using a Whatman No.1 filter paper, and subsequently subjected to a mild treatment with sodium sulfide (0.2 mg/ml) for 2 hours. The hair was recovered using a Whatman No.1 filter paper, rinsed with deionised water until free from residual sulfide. The recovered hair was dried at ambient conditions. The effects of immunisation on microbial keratinolytic activity was determined by incubating the immunised bovine hair (10 mg) with 0.6 ml of the isolated crude enzyme as described in Section 4.2.8.1 for 48 hours.

4.2.9 Analysis of the physical and biochemical changes

The biophysical transformation of the hair structure, as a result of the effects of treatment with the isolated crude microbial enzyme, was monitored and the extent of the damage assessed using scanning electron microscope (SEM) as described in Section 2.2.2.1.1.

4.3 Results and discussion

4.3.1 Laboratory studies on microbial biodegradation of bovine hair

The decomposition of keratinous substrates have been attributed to the production of specific, mostly extracellular proteolytic enzymes called keratinases, whose secretion is induced by the presence of the keratin substrate (Onifade *et al.*, 1998; Takiuchi *et al.*, 1982; Siesenop and Bohm, 1995). The presence of keratinolytic micro-organisms in soil have been reported (Macedo *et al.*, 2005; Tatineni *et al.*, 2008; Wainwright, 1982; Watson, 1956). The results of the laboratory-scale incubation of hair with soil, to demonstrate the capability of micro-organisms to biodegrade bovine hair, are illustrated in Figures 4.2 – 4.4 and 4.6 – 4.11. The SEM images are representative of the physical changes observed on the test samples of the brown, black and white bovine hair. Figure 4.1 represents the physical structure of the control sample. From the results the initial physical changes attributed to microbial biodegradation of hair were observed after 15 days (Figure 4.2). These changes were characterised by the abrasive or erosive appearance of the cuticle surface particularly at the basement region, typical of enzymatic degradation as previously observed with the commercial enzyme, proteinase K (Figure 3.14, Chapter 3).

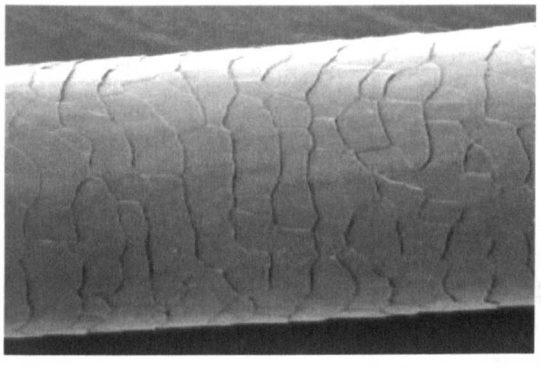

Figure 4.1: SEM image of intact hair shaft (mag. x1.0K, 5.0kV).

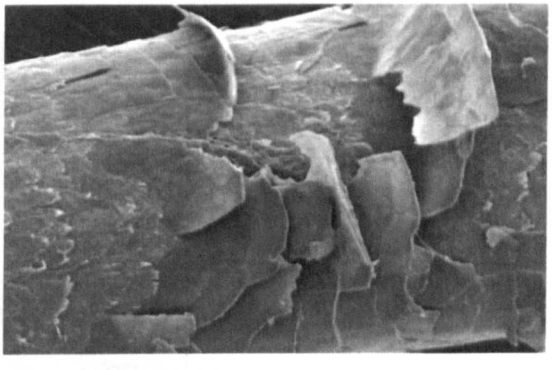

Figure 4.2: SEM image of the hair shaft illustrating early stages of degradation (mag. x1.0K, 5.0kV).

There was further evidence that the attachment or intercellular region between the cuticle/cuticle layers was preferably being degraded leading to weakening of the individual cuticle cells (Figure 4.3). Prolonged incubation was often marked by extensive erosion of the hair shaft suggesting many cuticle cells had undergone some form of degradation and fallen off (Figure 4.4.).

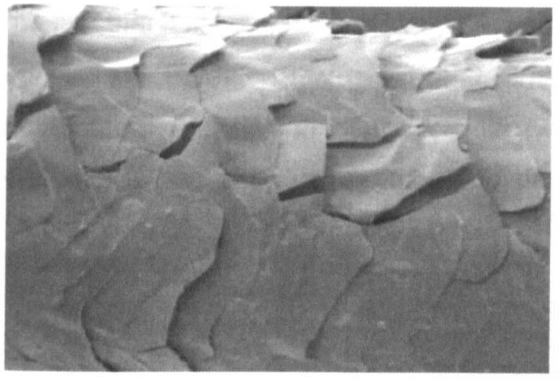

Figure 4.3: SEM image of the hair shaft illustrating weakening between cuticle cells (mag. x1.0K, 5.0kV).

Figure 4.4: SEM image illustrating microbial damage to the hair surface after 25 days of incubation (mag. x1.0K, 5.0kV).

Figure 4.5 is an illustration of a cross-section of the cuticle, which has been used as an aid to understanding the progressive mechanism of microbial degradation of the hair structure. The weakening and flaking of the cuticle suggests the initial point of attack was the intercellular cell membrane or cell membrane complex. The cell membrane complex (CMC) (Figure 4.5) is the component that provides attachment between the cuticle/cuticle layers and cuticle and cortex (Jones, 2001; Jones and Rivett, 1997).

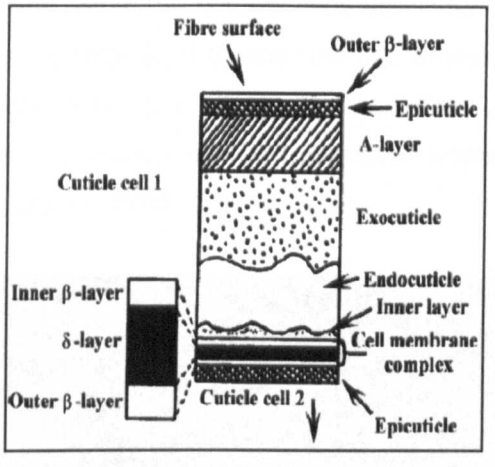

Figure 4.5: An illustration of a cross-section of the cuticle (Swift and Smith, 2001).

The observed mode of attack was comparable to the previously observed mechanism by the commercial enzyme, proteinase K (Section 3.5.7.1, Chapter 3). On the basis of the protein constituents of the cuticle, the most vulnerable region at which the attack could be initiated is the δ-layer of the cell membrane complex. The vulnerability of the δ-layer can be explained by

the fact that it is composed of proteinaceous material with low disulfide bonds (Allen *et al.*, 1991; Jones and Rivett, 1997; Swift and Smith, 2001). Degradation of the δ-layer caused the detachment and flaking of the cuticle (Figure 4.6), leading to an attack on the underlying endocuticle region, which is known to be susceptible to proteolytic degradation (Birbeck and Mercer, 1957; Fraser *et al.*, 1972; Orwin, 1979).

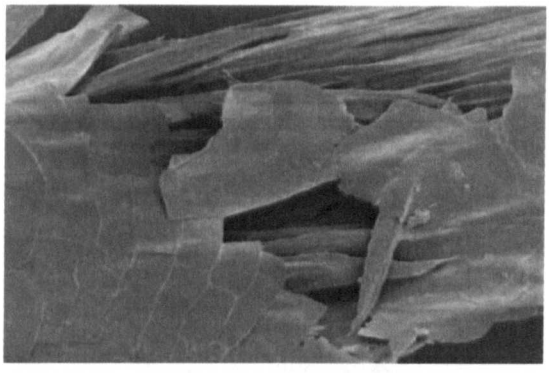

Figure 4.6: SEM image of the hair shaft illustrating flaking of the cuticle and fibrillation of the cortex after 25 days of incubation (mag. x1.0K, 5.0kV).

There was selective degradation within the cortical cells as evidenced by fibrillation of the fibres (Figures 4.6 & 4.7). This can be explained by the variable protein constituents and degree of crosslinking within the cortical cells. The vulnerable constituents of the cortex include the intercellular material that binds the macrofibrils, cytoplasmic remnants, and the less crosslinked orthocortex (Baumann, 1979; Jones, 2001; Orwin, 1979).

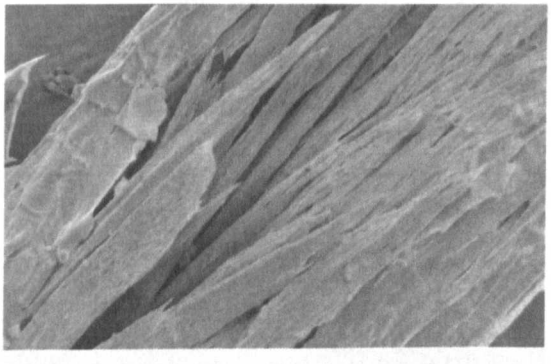

Figure 4.7: SEM image of the hair shaft illustrating fibrillated cortex after 30 days of incubation (mag. x1.0K, 5.0kV).

The fibrillation is indicative of the proteolytic digestion of these components, leaving the highly keratinised fibres (Figure 4.7), which compares well with the previously observed action of the two combined commercial enzymes in Chapter 3 (Section 3.5.7.3). The medulla was also observed to undergo preferential proteolytic degradation leaving the highly crosslinked cortical fibres (Figure 4.8). The observed selective degradation of the non-keratinised and less keratinised components of the hair both suggested the presence of proteolytic and keratinolytic activities. Importantly, it demonstrates a synergistic effect between proteolytic and keratinolytic microbial enzymes as the more likely fundamental mechanism by which micro-organisms enhance hair digestion.

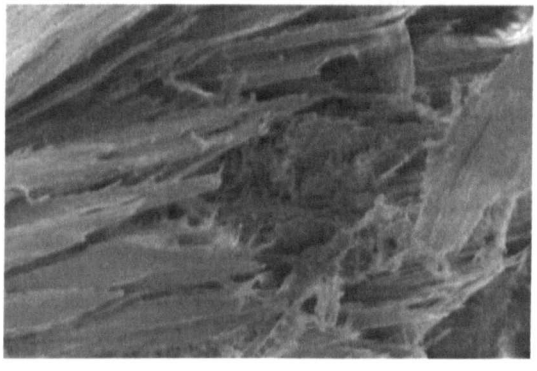

Figure 4.8: SEM image illustrating microbial damage to the medulla after 30 days of incubation (mag. x1.0K, 5.0kV).

In addition to enzymatic processes mechanical transformations of the hair structure were observed. These were characterised by tunnelling and splitting of the hair shaft (Figures 4.9 – 4.11), generally attributed to fungal mechanical keratinolysis as a result of mycelial penetration of the hair shaft (Degaetano *et al.*, 1992; Figueras *et al.*, 1997; Malviya *et al.*, 1992). The mechanical degradation by tunnelling and splitting of the hair shaft were random and non-selective compared to the enzymatic degradation.

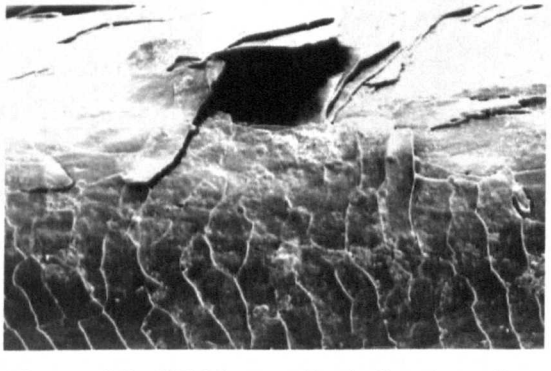

Figure 4.9: SEM image illustrating tunneling of the hair shaft (mag. x1.0K, 5.0kV).

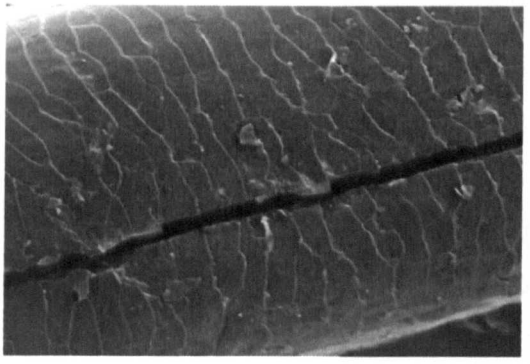

Figure 4.10: SEM image illustrating early stages of mechanical damage of the hair shaft (mag. x1.0K, 5.0kV).

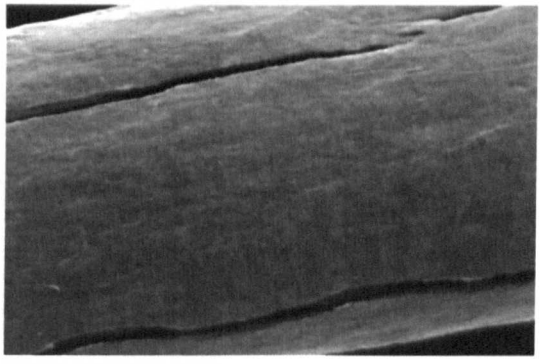

Figure 4.11: SEM image illustrating mechanical damage of the hair shaft after 30 days of incubation (mag. x1.0K, 5.0kV).

4.3.2 Optimum growth conditions

The keratinolytic micro-organism was observed to grow under aerobic conditions within the temperature range of 40 – 50°C and pH 11. Extensive degradation of hair sampled and examined during the growth period, was observed after 48 hours under the experimental growth conditions (Figures 4.12 – 4.13). The microbial degradation of keratin was further evidenced by the cloudiness and dye released from the Keratin azure substrate incubated with an aliquot from the culture of test organism (Figure 4.14). Growth period longer than 48 hours was observed to cause no additional effect on the substrate. The transformation of the substrate confirmed that the micro-organism was able to utilise the keratin substrate as a source of nutrients (carbon and nitrogen) to support growth.

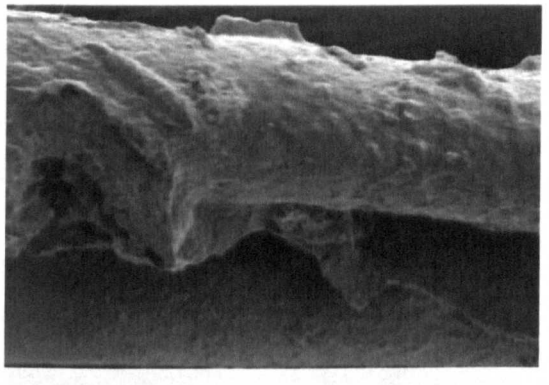

Figure 4.12: SEM image illustrating the decomposition of the hair shaft following incubation with the microbial growth culture (mag. x1.0K, 5.0kV).

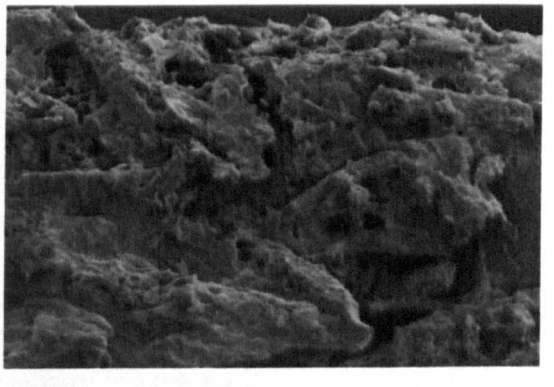

Figure 4.13: SEM image of hair degraded by the microbial growth culture (mag. x1.0K, 5.0kV).

Figure 4.14: An illustration of the degradation of keratin azure following 24 hours incubation at 50°C with an aliquot from the crude microbial culture. SB (blank: keratin azure with buffer), S1 & S2 (keratin azure with aliquot culture sample).

4.3.3 Isolation and identification of the keratinolytic micro-organism

A number of bacterial strains capable of degrading keratinous substrates have been isolated and reported (Gupta and Ramnani, 2006; Kim *et al.*, 2001; Riffel and Brandelli, 2006; Williams *et al.*, 1990). In this study, the isolated micro-organism was shown to be Gram positive with microscopic round rod-shaped cells (Figure 4.15). The micro-organism produced growth of a single colony with distinctive blue colour surrounded by egg-yolk coloured precipitate zones on the plate containing selective nutrient agar (Figure 4.16). Based on growth characteristics, cell morphology, as well as colony morphology the results indicated the micro-organism was a bacterium of the genus *Bacillus*.

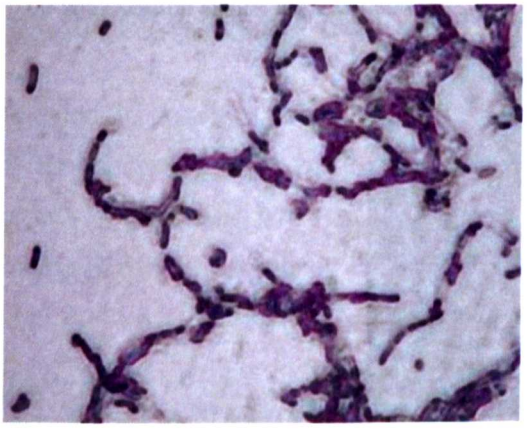

Figure 4.15: Light microscopy images of Gram positive bacterium isolated from the growth culture (mag. x100).

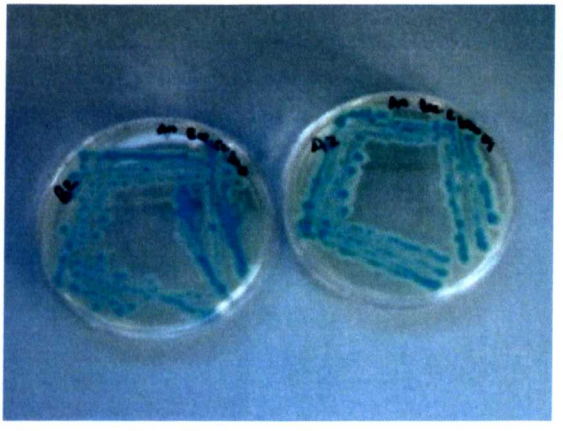

Figure 4.16: Photographic images showing growth of single colonies of the micro-organism, with visible blue colour surrounded by zones of egg-yolk coloured precipitates.

4.3.4 Microbial enzyme assays

4.3.4.1 Keratinolytic activity

Figure 4.17 represents the activity profile of the crude enzyme extract assayed with keratin azure as a substrate. The enzyme extract exhibited activity under a broad pH range of 7.5 – 10. Further examination found that the enzyme was more active in the alkaline pH range with the optimum activity determined at pH 9.0. The pH of optimum activity was evidenced by the intensity of the dye released from the substrate (Figure 4.18), with the calculated enzyme activity of 50 Units/ml. In comparison the enzyme activity at pH 7 was 19 Units/ml, pH 7.5 was 29 Units/ml, while pH 8 and 10 were 40 and 31 Units/ml, respectively.

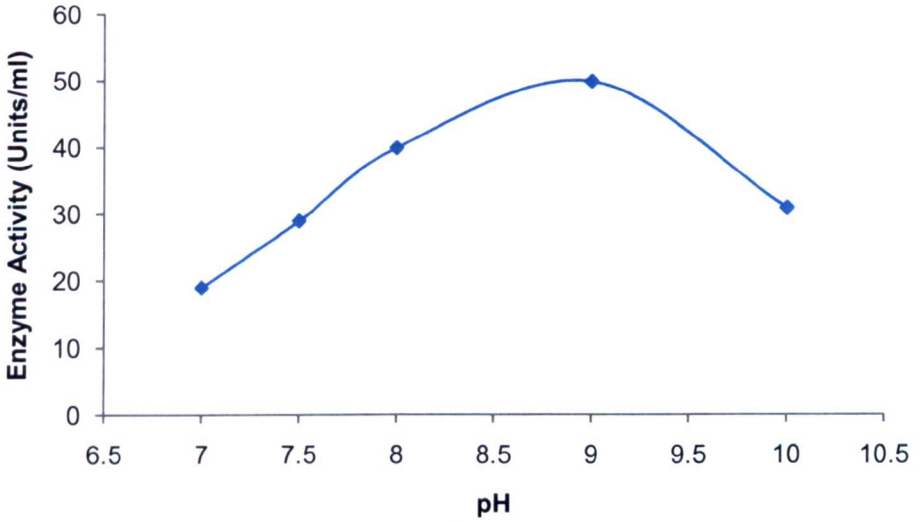

Figure 4.17: Keratinolytic activity of the isolated microbial crude enzyme studied in the pH range 7.0 – 10.0 with keratin azure as a substrate.

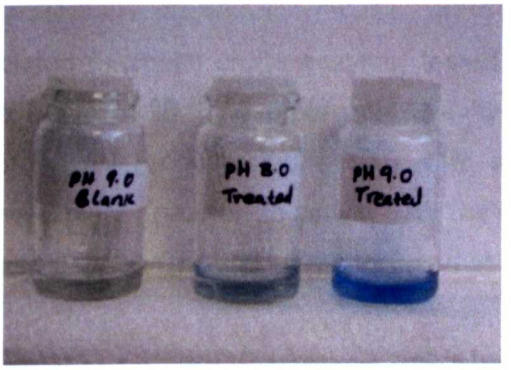

Figure 4.18: A representation of a pH range for optimum activity evidenced by the intensity of soluble dye released following incubation of keratin azure with the isolated microbial crude enzyme at 50°C.

The microbial keratinase was observed to be active in the temperature range of 40 – 60°C, with the optimum activity at 50°C (Figure 4.19). The following results, therefore, established the optimum range of working conditions for the isolated crude enzyme as pH 9.0 and 50°C. This was found to be consistent with other evidence related to reviews on serine proteases, reported to be active at a neutral and alkaline pH range, with an optimum range between pH 7.0 and 11.0, and optimal temperature of activity up to 60°C (Horikoshi, 1996; Rao *et al.*, 1998).

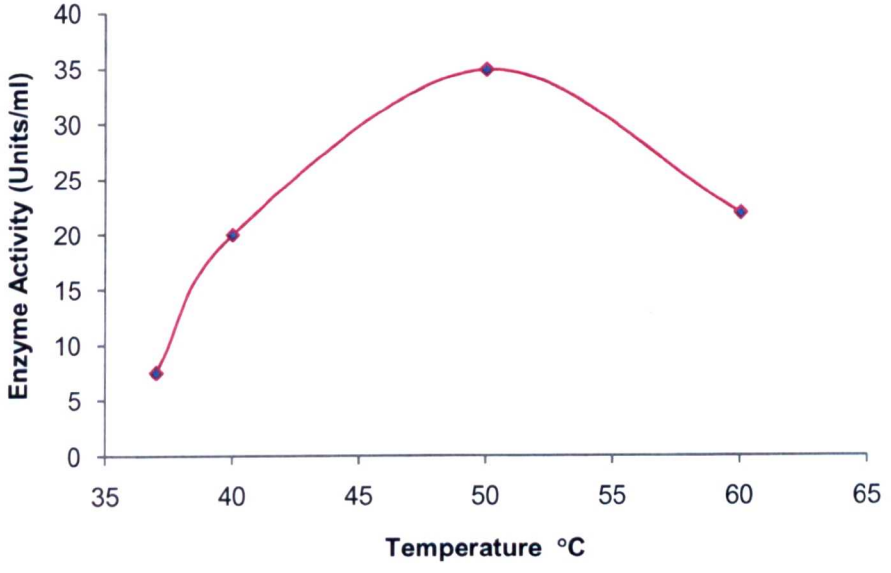

Figure 4.19: Keratinolytic activity of microbial crude enzyme extract studied at pH 9.0 in the temperature range of 37 – 60°C with keratin azure as a substrate.

4.3.4.2 Proteolytic activity

Proteolytic activity of the isolated microbial crude enzyme, tested with Hide powder-Remazol Blue substrate, is shown in Figure 4.20. The proteolytic activity of the crude enzyme was similarly observed over a broad pH range of 7.0 – 10.0, with an optimum at pH 9.0. The microbial crude enzyme showed higher proteolytic activity with the Hide powder as a substrate, with the maximum enzyme activity of 90 Units/ml being achieved at pH 9.0.

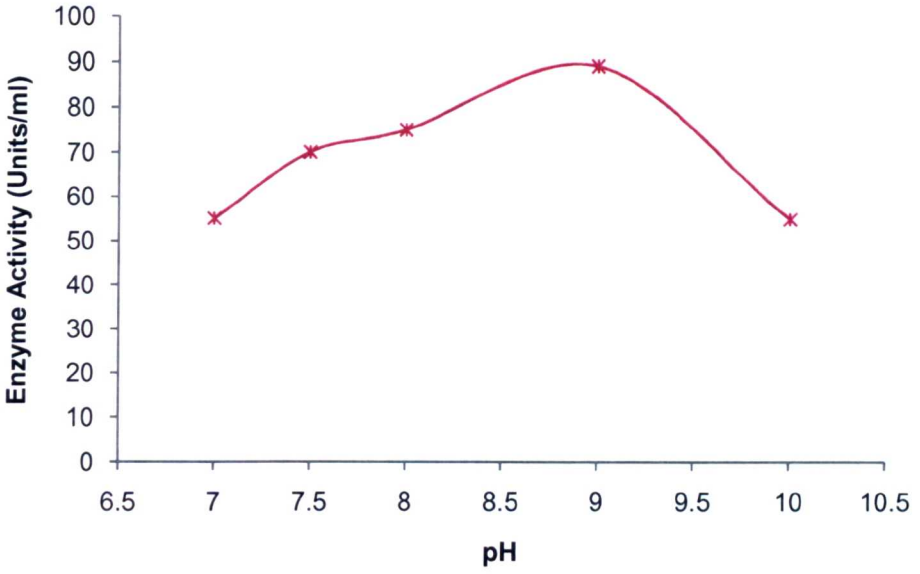

Figure 4.20: The proteolytic activity of the isolated microbial crude enzyme studied at the pH range 7.0 – 10.0 using Hide Powder-Remazol Brilliant Blue as a substrate.

4.3.4.3 Effect of inhibitors

The keratinases from *Bacillus* have been widely studied, and are known to belong to the subtilisin family of serine proteases (Brandelli, 2008; Gupta and Ramnani, 2006; Onifade *et al.*, 1998). In this study the isolated crude enzyme was proved to be inhibited by E.D.T.A. (Figure 4.21), indicating the microbial enzyme extract was a metalloprotease. The serine-type metalloproteases from bacterial species of *Bacillus* and other sources have also been reported (Sousa *et al.*, 2007; Thys and Brandelli, 2006). The crude keratinolytic enzyme was, however, not inhibited by 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF).

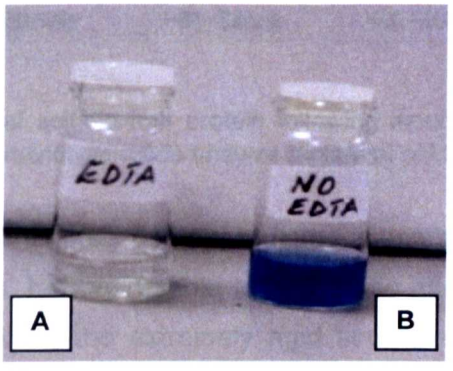

Figure 4.21: A representation of the effect of E.D.T.A. as a protease inhibitor following incubation of keratin azure with the isolated crude enzyme at pH 9.0 and 50°C for 24 hours. (A) E.D.T.A. treated, (B) No E.D.T.A.

4.3.5 Hydrolysis of bovine hair substrates

Mammalian hair keratins are well known for their high resistance to proteolytic enzymes, being stabilised by their high degree of crosslinking of disulfide bonds. However, it is also known that keratinases have the capacity to degrade keratin substrates (Fraser *et al.*, 1972; Onifade *et al.*, 1998). The ability of the isolated microbial crude enzyme to degrade native keratin, tested using bovine hair as a substrate, is represented by the time course graph of solubilised protein shown in Figure 4.22. The degradation of keratin, followed by measurement of soluble protein, shows that the average concentration of soluble protein detected in the initial hour of incubation was 10 ± 0.6 μg/ml, increasing to 65 ± 3 μg/ml after 10 hours of incubation. During this period the solubilisation of the hair substrate was characterised by a linear phase of slow release of soluble proteins with an average initial reaction rate of 0.11 ± 0.02 μg/ml min^{-1}.

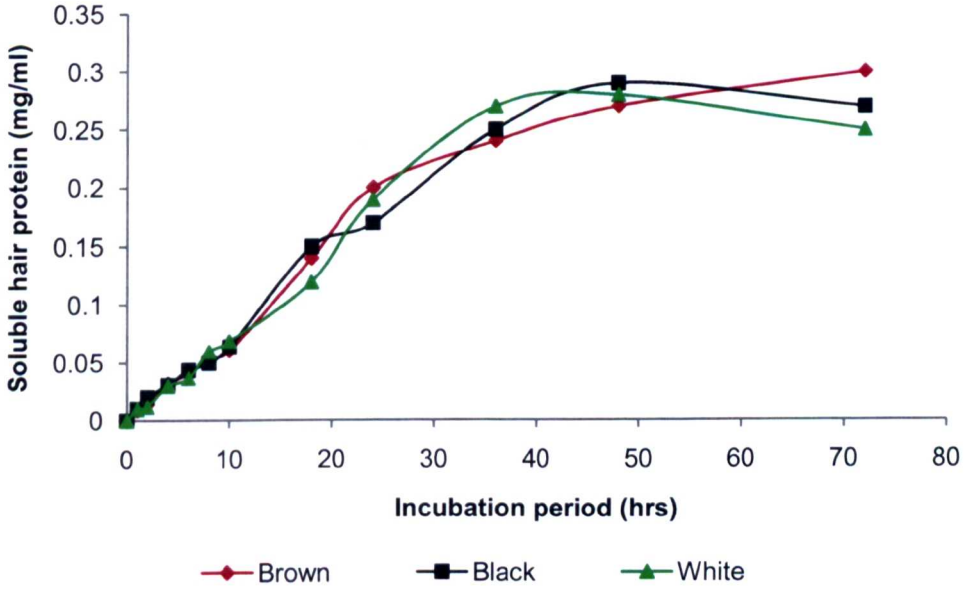

Figure 4.22: The concentration of soluble hair protein following incubation of brown, black and white bovine hair substrates with the keratinolytic crude enzyme isolate at pH 9.0 and 50°C for 0 – 72 hours.

It is proposed that in the first 10 hours when degradation appears to be slow, the substrate was less accessible and, therefore, less vulnerable to enzymatic attack. This initial phase of slow degradation may be explained by the extremely rigid structure of keratins (Bradbury, 1973; Fraser *et al.*, 1972). Thus, it may be assumed that this initial period was the structural opening phase, the point at which the keratinase was attacking the cell membrane of the cuticle cells.

Following an attack to the cuticle the keratin substrate became accessible, and subsequently, vulnerable to proteolytic enzymes which may be present in the crude enzyme. This may be supported by the fact that degradation appears to progress rapidly thereafter, with an average of 187 ± 3 μg/ml soluble protein being detected in 24 hours, representing a 3 fold increase in the concentration of soluble protein. The highest concentration of soluble protein was detected after 36 and 48 hours, with an average of 250 μg/ml and 280 μg/ml of soluble protein detected, respectively. However, no significant increase in the concentration of soluble protein realised after 48 hours.

Substrate specificity is an important characteristic feature of enzymatic reactions (Palmer, 1981, Voet *et al.*, 2008). In comparing the degradation of the substrates (brown, black and white hair), similar profiles was observed (Figure 4.22), suggesting similarities in enzyme specificity. This further demonstrates that different bovine hairs are similar in their chemical constituents, hence undergoes similar hydrolytic modification by the isolated microbial crude enzyme.

4.3.5.1 Hydrolysis of non-keratin substrate

Figures 4.23 – 4.25 illustrate the ability of the microbial enzyme extract to hydrolyse non-keratinous substrate such as bovine skin substrate. The isolated crude enzyme was shown to hydrolyse raw bovine skin demonstrating its diverse substrate specificity. Although substrate specificity is a fundamental characteristic of enzymes, most proteases exhibit diverse specificity, having the capability to target multiple substrates (Lopez-Otin and Bond, 2008; Palmer, 1981). Figure 4.23 shows the samples of hydrolysed bovine skin, evidenced by the cloudy solution (Samples 2a & 2b), following proteolytic action by the isolated microbial enzyme. Figures 4.24 and 4.25 represent the control and the test samples, respectively.

Figure 4.23: A representation of hydrolysis of raw bovine skin by the isolated microbial crude enzyme. Incubation was at pH 9.0 and 50°C for 72 hours. (1) Control, no enzyme, (2a & 2b) Enzyme treated.

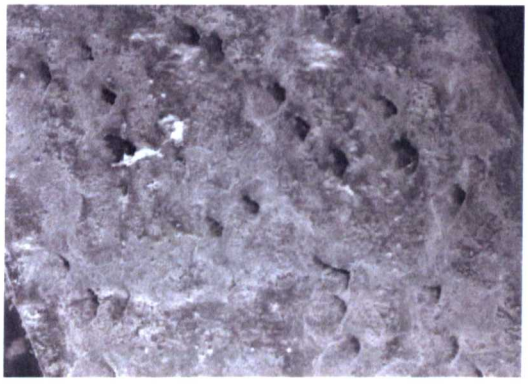

Figure 4.24: SEM image illustrating the surface of intact raw bovine skin (mag. x100, 5.0kV.

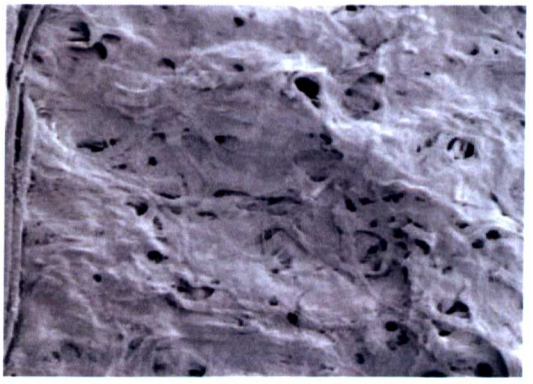

Figure 4.25: SEM image illustrating digested raw bovine skin following incubation with the isolated microbial enzyme at pH 9.0 and 50°C for 72 hours (mag. x100, 5.0kV.

4.3.5.2 Modification of the hair structure by the microbial enzyme

Figures 4.26 – 4.31 represent the microscopy (SEM) images of the physical morphological characteristics of bovine hair samples following incubation with the isolated microbial crude enzyme. As evidenced by the microscopy images presented, the physical transformation of the hair substrates by the crude enzyme was comparable to the previously observed action by the commercial enzymes, proteinase K, and proteinase K plus trypsin (Sections 3.5.7.1 & 3.5.7.3, Chapter 3), suggesting similarities in their mode of action. In the first hour of incubation with the

crude keratinolytic enzyme, the hair surface, specifically the cuticle was characterised by erosion and slight lifting (Figure 4.26), which was attributed to the degradative mechanism of the enzyme as previously observed by the action of commercial enzymes in Chapter 3. The erosion and lifting of the cuticle cells was indicative of enzymatic attack on the intercellular layer, the cell membrane complex (CMC), which was considered a key step in the mechanism of hair degradation since it involved opening the complex structure from the outer surface of its most resistant and highly crosslinked component. As evidence of preferential degradation of the cementing intercellular region (CMC), Figure 4.27 shows delaminated cuticle cells being degraded from the inner side of the endocuticle region. It is suggested that this action by the keratinolytic enzyme, improved accessibility to the inner components of hair and the degradation action by proteolytic enzymes.

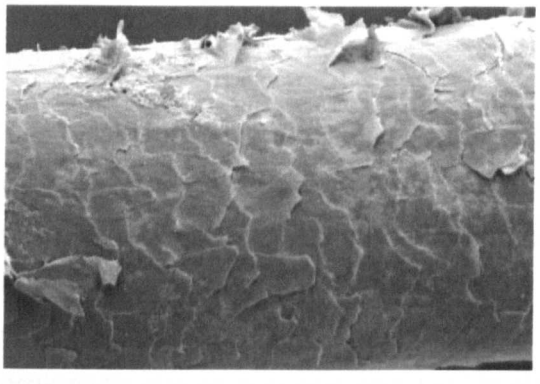

Figure 4.26: SEM image illustrating damage to the hair shaft following incubation with the isolated microbial enzyme at 50°C for 1 hour (mag. x1.0K, 5.0kV).

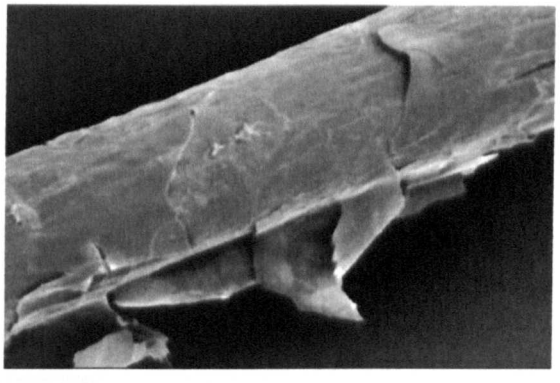

Figure 4.27: SEM image illustrating damage to the hair shaft following incubation with the isolated microbial enzyme at 50°C for 24 hours (mag. x1.0K, 5.0kV).

The efficiency and proteolytic ability of the crude enzyme to decompose the non-keratinous components of hair is demonstrated by Figure 4.28. The microscopy image shows evidence of the structure of the dissolved medulla. The dissolution of the medulla occurred within 24 hours of incubation. The disintegration of other hair components progressed with incubation time, and it is believed that the isolated microbial enzyme highly enhanced this process by its ability to degrade both the keratinised and non-keratinised hair constituents. As shown in Figure 4.29, splitting and fibrillation of the cortex was clearly visible after 36 hours of incubation. The structural characteristics of the substrate (hair) at this point, compares well with the structural modification by the action of the combined commercial enzymes observed after 72 hours of incubation (Figure 3.22, Chapter 3). This structural modification further strongly demonstrates substrate diversity of the isolated microbial enzyme. Substrate diversity of extracellular microbial

proteases is an important property for commercial exploitation in protein degradation in various industrial processes (Horikoshi, 1999; Kumar and Takagi, 1999; Rao *et al.*, 1998).

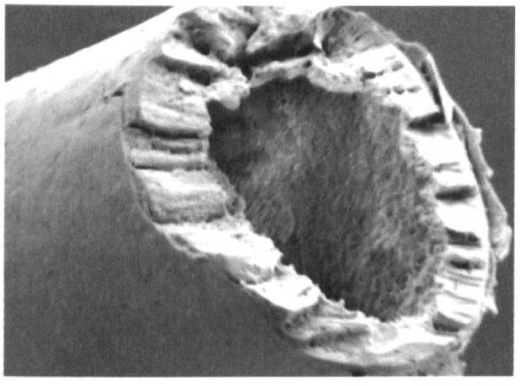

Figure 4.28: SEM image illustrating damage to the medulla following incubation with the isolated microbial enzyme at 50°C for 24 hours (mag. x1.0K, 5.0kV).

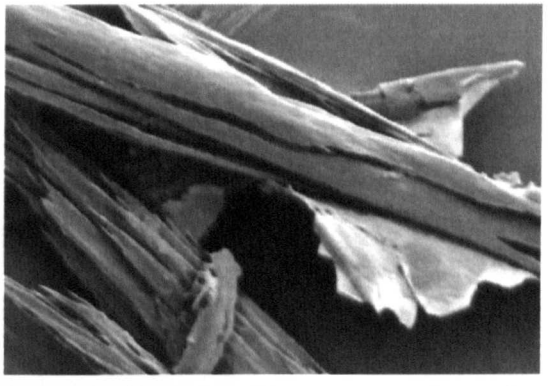

Figure 4.29: SEM image illustrating disintegration hair following incubation with the isolated microbial enzyme at 50°C for 36 hours (mag. x1.0K, 5.0kV).

The effectiveness of the isolated microbial enzyme and its ability to enhance the decomposition of hair was further demonstrated in Figures 4.30 and 4.31, respectively. As illustrated by the microscopy images complete physical disintegration of the hair structure was evident after 48 hours of incubation with the isolated microbial enzyme (Figure 4.30). The enhanced disintegration of hair is proposed to have been the result of the cooperative action between the proteolytic and keratinolytic enzymes in a synergistic manner.

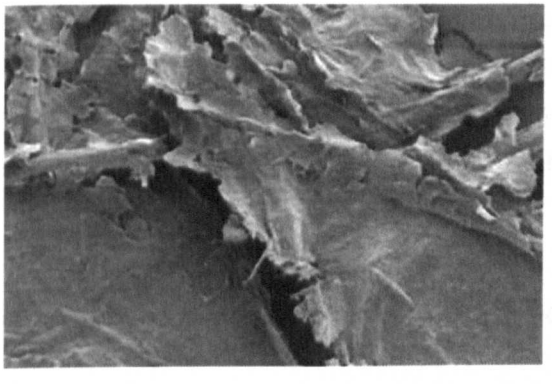

Figure 4.30: SEM image illustrating the disintegrated cuticle following incubation with the isolated microbial enzyme at 50°C for 48 hours (mag. x1.0K, 5.0kV).

However, the evidence shows that the cuticle and the highly keratinised cortical fibres were not completely degraded within the time scale of the experiment. As shown in Figure 4.31 the cuticle and some cortical fibres still existed, but as disordered fragments after 72 hours of incubation.

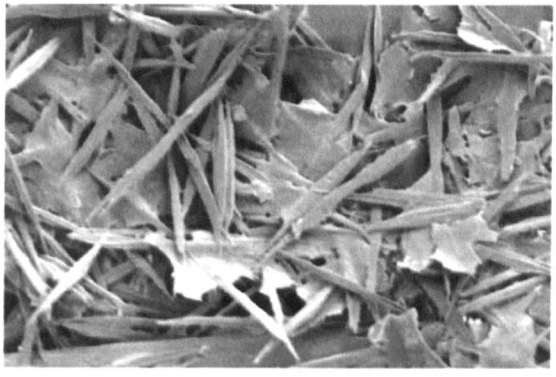

Figure 4.31: SEM image illustrating the fragmented hair structure following incubation with the isolated microbial enzyme at 50°C for 72 hour (mag. x1.0K, 5.0kV).

The similarity of these structures with the enzyme-chemically pretreated hair samples (Figures 3.24 & 3.25, Chapter 3) indicate that the isolated microbial enzyme may be an effective and efficient replacement for the chemical pretreatment process and commercial enzymes for the decomposition of hair. The application of microbial keratinolytic enzymes in industrial processes converting fibrous proteins such as feathers, nails, horns and hooves into useful biomass and specific amino acids have been reported, especially in the biotechnological production of animal feed supplements (Gupta and Ramnani, 2006; Kida *et al.*, 1995; Onifade *et al.*, 1998; Papadopoulos, 1986). In this study, the observed action of the identified micro-organism and its extracellular enzyme represent an attractive alternative to enhance the biodegradation of hair during composting.

4.3.6 Effect of immunisation on enzymatic hydrolysis of bovine hair

Immunisation of hair by lime, $Ca(OH_2)$, pretreatment is a well known phenomenon in the leather industry. The pretreatment with lime is known to change the character of hair keratin, and renders it resistant to the reducing action of sulfide, by converting the cysteinyl residue of hair to a new amino acid known as lanthionine, a stable mono-sulfide residue which is not cleaved by sulfur-nucleophiles (Covington, 2009; Heidemann, 1993; Merril, 1956; Windus and Showell,

1968). Hair immunisation is the principal basis for the practise of 'hair-save', in which bovine hair is recovered during the industrial unhairing process. Altering the chemical character of a particular substrate may affect the way by which it is acted upon by an enzyme, and the substrate may develop complete resistance to enzymatic attack.

Figure 4.32 represents the profile of the average concentration of soluble hair protein detected during incubation of immunised bovine hair substrates with the isolated microbial crude enzyme. As shown, the microbial enzyme was able to hydrolyse immunised bovine hair, with an average concentration of soluble protein detected 23 ± 3 μg/ml in the first hour, increasing to 110 ± 2 μg/ml of protein in 10 hours of incubation.

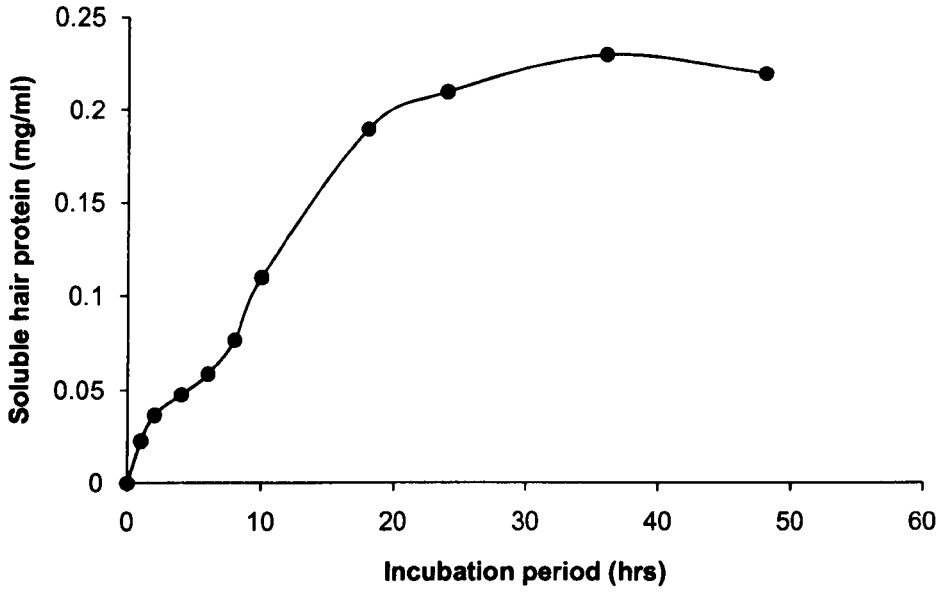

Figure 4.32: The average concentration of soluble hair protein following incubation of immunised bovine hair substrate with the isolated microbial crude enzyme at pH 9.0 and 50°C for 0 – 48 hours.

The protein concentration detected for immunised hair in the first hour and after 10 hours was approximately double the concentration of soluble protein detected for the intact hair samples (control) in the same period of incubation (*i.e.* 10 μg/ml and 65 μg/ml, respectively). There is the possibility that pretreatment of bovine hair with lime for 18 hours to cause immunisation may have improved enzyme accessibility, either by swelling the structure, or hydrolysis of the non-keratinous proteins following prolonged exposure to alkaline conditions. However, the

concentration of soluble protein detected after 36 and 48 hours were comparable to non-immunised hair substrates, yielding 230 and 220 µg/ml of protein, respectively.

The results from this study reveal that immunisation did not inhibit the keratinolytic action of the isolated microbial crude enzyme, which further demonstrated its substrate diversity. In addition, the results strongly demonstrate the potential use of the isolated microbial enzyme in bioconversion of keratinous substrates such as tannery hair waste.

4.3.6.1 Structural modification of immunised hair by the microbial enzyme

Structural changes showing the degradation of immunised bovine hair substrate with the isolated microbial enzyme are represented by the microscopy (SEM) images, Figures 4.34 – 4.38. Figure 4.33 is the control sample (immunised and sulfide treated hair sample). Figure 4.34 represents the morphological changes of the hair surface following the initial stages of incubation with a keratinolytic enzyme. The surface was typically characterised by the patchy erosion of the cuticle, indicating the effects of the enzymatic action.

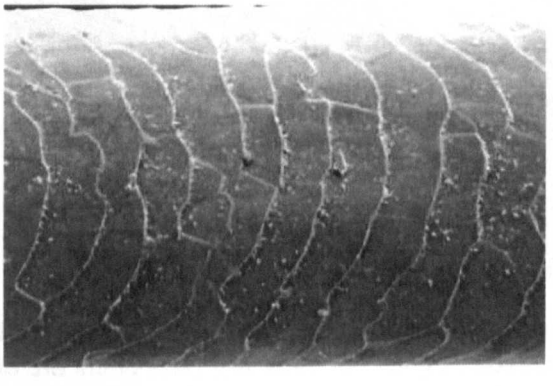

Figure 4.33: SEM image of immunised hair shaft (mag. x1.0K, 5.0kV).

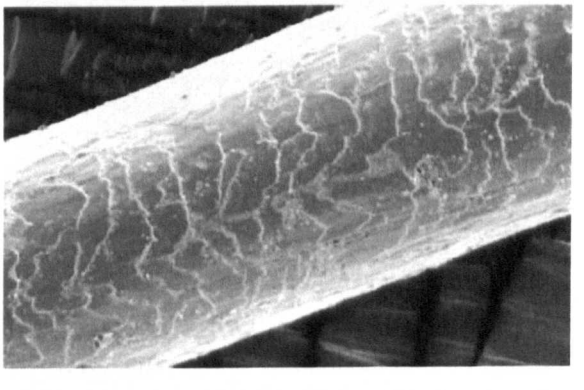

Figure 4.34: SEM image illustrating the damage to the immunised hair shaft following incubation with the isolated microbial enzyme at 50°C for 1 hour (mag. x1.0K, 5.0kV).

Whereas the cuticle presented resistance to enzymatic degradation, the medulla and part of the cortex appear to have been dissolved after 24 hours (Figure 4.35). However, after 36 hours of incubation, most of the cuticle had been degraded (Figure 4.36). As shown, (Figures 4.37 & 4.38), prolonged incubation with the enzyme (36 – 48 hours) resulted in extensive structural

damage, which further demonstrates the considerable biotechnological potential of the isolated microbial enzyme.

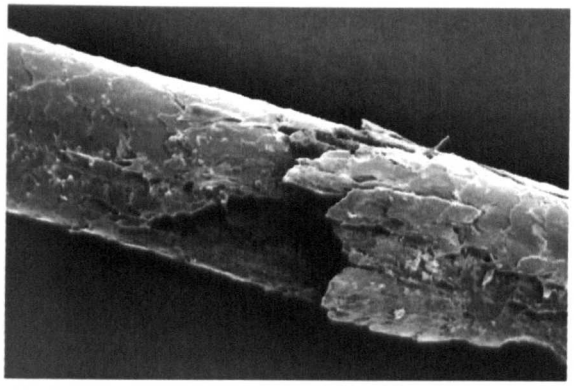

Figure 4.35: SEM image illustrating degraded medulla and cortex of immunised hair following incubation with the isolated microbial enzyme at 50°C for 24 hours (mag. x1.0K, 5.0kV).

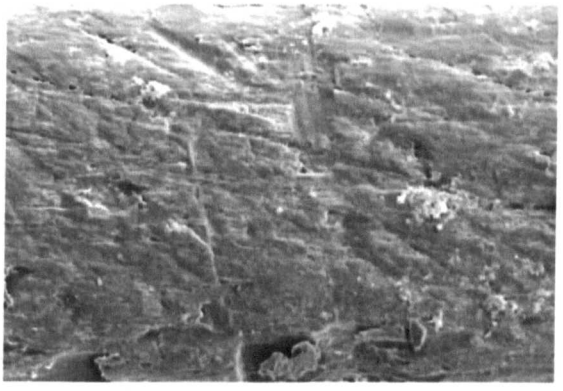

Figure 4.36: SEM image illustrating the damage to the cuticle surface of immunised hair following incubation with the isolated microbial enzyme at 50°C for 36 hours (mag. x1.0K, 5.0kV).

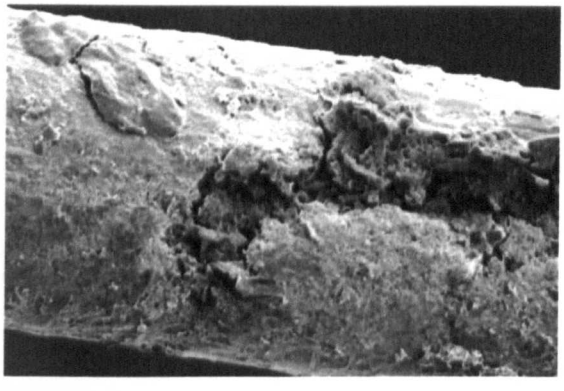

Figure 4.37: SEM image illustrating the damage to the immunised hair shaft following incubation with the isolated microbial enzyme at 50°C for 36 hours (mag. x1.0K, 5.0kV).

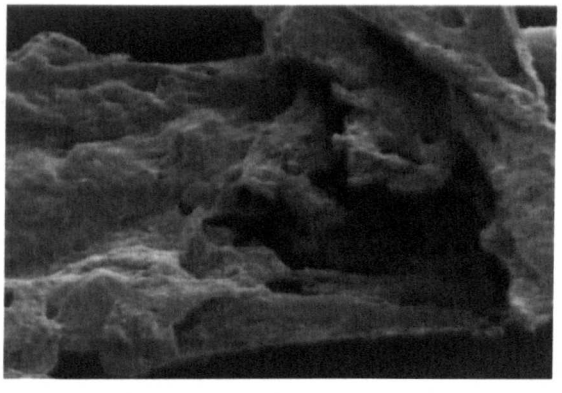

Figure 4.38: SEM image illustrating the disintegration of the immunised hair structure following incubation with the isolated microbial enzyme at 50°C for 48 hours (mag. x1.0K, 5.0kV).

4.3.7 Practical parameters for the application of the microbial enzyme

The laboratory experimental results have demonstrated the capability of the specific bacterium of the *Bacillus* species to efficiently decompose bovine hair substrates. Subsequently, a natural system for the decomposition of bovine hair may, therefore, be designed based on the optimised experimental conditions. From the bioconversion technical point of view, the design of

reaction conditions for the enhancement of microbial degradation of bovine hair is proposed to include the following fundamental parameters:

1. Setting appropriate general environmental conditions to sustain the growth of the specific bacterial species responsible for degrading hair and facilitate their metabolic efficiency. These conditions were identified as: (i) the substrate, as a source of carbon and nitrogen, (ii) temperature, (thermophilic range, 40 – 50°C), and (iii) presence of moisture, preferably within the range of 53 ± 4% moisture content.

2. The keratinolytic ability of the bacterium may be exploited by applying the isolated microbial crude enzyme as an inoculant to enhance the biodegradation of hair during the composting process. The inoculant, containing a protein concentration of 9 mg/ml, may be applied at the ratio of 1:17 (volume of enzyme to substrate, as used in this study). The proposed range of pH of activity is 7.5 – 10.0, preferably pH 9.0, and temperature range of 40 – 60°C, preferably at 50°C.

These parameters are, therefore, proposed as the working conditions for the composting process, discussed in Chapter 5. Any changes to the proposed parameters will depend on the compatibility with the overall performance of composting process.

4.4 Summary of the Chapter

- The laboratory incubation studies have demonstrated the presence of micro-organism with the capability to efficiently degrade bovine hair. Under the controlled experimental conditions of 40°C, and soil moisture content of 53 ± 4%, the biodegradation of hair was observed to involve both enzymatic and mechanical processes. The enzymatic process was characterised by the erosion of the fibre surface (the cuticle layer), intercellular degradation, and fibrillation of the hair structure. In contrast, the mechanical degradation was attributed to fungal attack, and was characterised by fibre surface colonisation by mycelial growth, tunnelling and splitting of the hair shaft. The degradation by enzymatic process was observed to be particularly selective with regard to the breakdown of the structural components of hair, whilst that of the mechanical process appeared to be random and non-selective.

- The biodegradation mechanism associated with microbial extracellular enzymes was observed to preferentially begin and proceed via the most vulnerable structural components as decomposition progressed with time. This followed the structural sequence of (1) δ-layer of the cell membrane complex, (2) endocuticle, (3) medulla, and (4) the cytoplasmic and fibrillar components of the cortex. The cuticle and the highly keratinised cortical fibres were, however, more resistant to degradation.

- The micro-organism responsible for hair degradation was isolated and identified as belonging to the Gram positive bacterium of the genus *Bacillus*. The micro-organism was able to grow aerobically in an alkaline culture media, pH 11, containing hair as the only source of carbon and nitrogen, at the temperature range of 40 – 50°C. Sampled hair showed extensive structural decomposition, providing evidence that the micro-organism was able to metabolise the crystalline α-keratin substrates.

- The microbial enzyme was partially purified and characterised as an extracellular keratinase, and an alkaline metalloprotease. The keratinase exhibited a broad range of pH of activity, 7.5 – 10, with an optimum at pH 9.0. Similarly, the keratinase was found to be active in the temperature range of 40 – 60°C, with an optimum at 50°C. The high temperature indicated good thermal stability, which is an advantage for the potential biotechnological application. The microbial keratinase was found to have a broad substrate specificity, hydrolysing keratin and raw bovine skin, thus demonstrating both keratinolytic and proteolytic potential. The microbial enzyme was highly effective in degrading hair, achieving complete disintegration of the structure in 48 hours of incubation. Thus, it offers the best alternative to chemical pretreatment and the use of commercial enzymes in the technology of bio-processing of hair waste.

- The laboratory studies, evidenced by the concentration of soluble protein and microscopy (SEM) images, showed that the isolated microbial enzyme was able to hydrolyse immunised hair substrates, which increases its potential biotechnological advantage in enhancing the degradation of tannery hair waste in a bioconversion system such as composting.

- Consequently, the use of the microbial keratinolytic enzyme or the micro-organism is proposed for a practical composting process.

CHAPTER 5

COMPOSTING OF TANNERY BOVINE HAIR WASTE

CHAPTER 5

5. INTRODUCTION

5.1 Management of tannery wastes and composting

Chapter one of this thesis highlighted some of the key economic and environmental aspects of the global leather industry. Briefly, the industry produces about 18 billion square feet of leather annually, at a value estimated at $ 40 billion and provides employment to 500,000 people worldwide. The industry also plays a major environmental role in recovering and adding value to the raw hides and skins, which are the by-products from the meat industry (COTANCE, 2002; ICT, 2009). Worldwide, the average wastewater discharge is more than 1.5 million litres per day, and solid waste generated from tannery processes is estimated to be 6 million tonnes per year (Rajamani *et al.*, 2009). The composition of tannery solid wastes generally includes fleshing, fats, hair, trimmings and shavings. The current disposal methods for tannery solid wastes are either by way of incineration, gasification, but more commonly by land treatment (Font and Esperanza, 1998; Hogg, 2007; Hughes, 1988; Martin, 1986). The tanning industry occupies a prominent place in the European Community's economy; with a share of 25% of the world's leather production and a trade turnover of about € 8 billion. The economic role notwithstanding, the tanning sector generates approximately 1 million tonnes of solid waste annually as a result of leather processing, which are mainly deposited in landfills (COTANCE, 2002). However, this is discouraged due to environmental and ecological reasons, and is not in fulfilment of the legislative objectives of the European Council Directive on landfilling of waste (Directive 99/31/EC) and the Waste Framework Directive (Directive 75/442/EEC).

The increasing environmental concern and stringent legislative discharge limits for tannery wastewaters and solid waste disposal have led to the development of 'hair-save' hair recovery techniques that avoid the dissolution of hair and subsequent discharge into the wastewaters. Consequently, the accumulation of large amounts of solid hair waste has been on the rise posing disposal problems (Frendrup, 2000; Germann, 1997). In order to minimise the negative environmental effects associated with land disposal, the European Union through the Landfill Directive (99/31/EC) has imposed limits on the amount of biodegradable waste that can be disposed to landfill whilst encouraging environmentally sound alternative

treatments such as composting. The use of waste hair and related keratinous wastes such as wool, and feathers or their by-products, as nutrient sources for crop production and dietary protein supplements have been considered attractive as 90% of the dry weight of the fibres consists of crude keratin protein, and bovine hair contains 15% nitrogen (Cantera and Buljan, 1997; Dalev, 1994; Fontenot *et al.*, 1983; Money, 1991; White *et al*, 1991). However, compared with other waste streams, the decomposition of these keratin-rich materials is difficult under natural conditions due to the presence of high content of disulfide crosslinks of the amino acid cystine (Onifade *et al.* 1998; Yamauchi *et al.*, 1996).

Despite the fact that the use of tannery hair waste as a source of organic fertiliser has been proposed as the most potential economically viable option for the management of the bulk of hair recovered during tanning (Cantera and Buljan, 1997; IUE, 2008; Money, 1991), there are no publications on the practicalities of utilisation of hair waste as a substrate for composting. Consequently, there is need for information about the biodegradation of hair in a composting environment. This section of research, therefore, forms part of the preliminary effort towards the development of a more practical approach to hair waste disposal through composting as opposed to landfilling and incineration, and reflects the overall EU waste management policy of resource conservation and sustainability.

5.2 Aims and Objectives

The overall aim of this chapter was to determine process operating parameters to enhance the decomposition of hair during composting. This section also represents the final and the applied part of this research. Chapter 4 of this research thesis demonstrated the presence of specific micro-organisms with the ability to effectly decompose hair. This chapter, therefore, will aim to exploit the biotechnological potential of the identified micro-organisms in order to improve the biodegradation of hair during composting. The key objectives are:

- To determine appropriate process conditions for composting hair.
- To collect process operational data for the evaluation of the composting process and the impact on the process performance.
- To evaluate the effects of adding a microbial inoculant possessing the capability of degrading hair with the aim of improving the decomposition of hair during the composting process.

- To monitor structural changes in substrate characteristics to evaluate the extent of biodegradation and relate to the process performance parameters.

- To analyse the physicochemical characteristics of the product.

5.3 Materials and methods

5.3.1 Composting raw materials

The feedstock materials for composting comprised intact bovine hair, dry leaves, soil, and wood chips/sawdust as bulking agent (Figure 5.1). The bulking agent and dry leaves were shredded manually to particle size range of 0 – 10 mm to provide porosity. The soil samples were sieved through a 2 mm sieve.

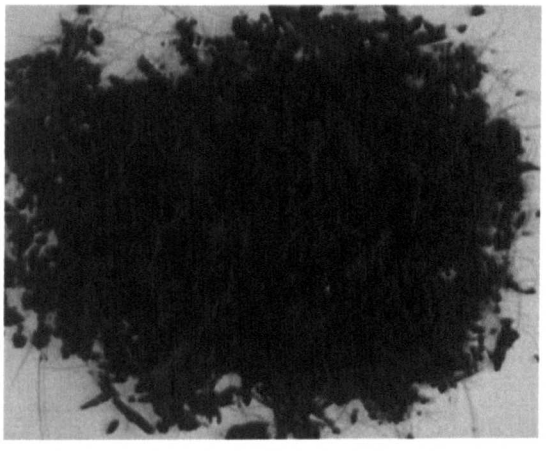

Figure 5.1: Physical characteristics of the raw feedstock mixture for composting.

5.3.2 Composting set-up and experiments

In order to establish appropriate conditions for composting two composting methods were studied: (1) Static laboratory-scale composting trial, and (2) Rotary Drum In-Vessel composting. The rotary drum composting comprised of two phases. In phase 1, which acted as the control experiment, the feedstocks were composted without the inoculant. In phase 2 composting was carried out using the inoculant containing the previously isolated microbial keratinase enzyme extract. The composting protocols are described below.

5.3.2.1 Laboratory-scale composting trial

The laboratory-scale composting trial was initially carried out in a 10 litre plastic container (Figure H1, Appendix H) designed according to Pagans *et al.* (2006). The schematic diagram of the container is shown in Figure 5.2. The inside chamber (A) was fitted with a 2

litre plastic carrier to support the feedstock. The plastic carrier was perforated with 0.2 cm (diameter) holes at the bottom to allow air circulation and leachate to flow. The lid and sides of the container were covered with woollen fabric material and aluminium foil to provide thermal insulation (5). The design was aimed at minimising heat losses to the surrounding as much as possible. Two holes were perforated in the lid of the container and a long stemmed thermometer (3), and air inlet tube (4) inserted, respectively.

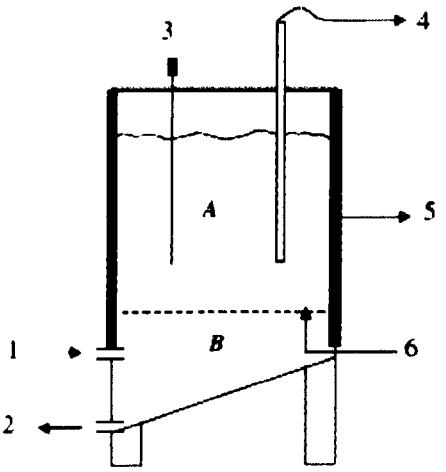

Figure 5.2: Schematic diagram of the laboratory-scale composting bin. (1) air inlet, (2) leachate outlet, (3) thermometer, (4) air inlet tube, (5) thermal insulation, (6) perforated carrier, (A) feedstock volume, (B) leachate collection.

The feedstock mixture (1.5 kg) contained bovine hair (0.025 kg), dry leaves (0.3 kg), soil (0.3 kg), and woodchips/saw dust (1:1), 0.875 kg. The moisture content of the feedstock was adjusted to 54 ± 2% at the start of the experiment. The initial pH of the mixture was 6.5, and a carbon to nitrogen ratio of 35:1. The composting trial was carried out in a ventilated incubator at 40 – 50°C under static conditions. For a period of 40 days, the feedstock was manually turned every 7 days, and the temperature, pH and moisture regularly monitored. The temperature readings were taken three times per day for each 3 days of the week and the average reported. The physical changes of the feedstock and structure changes of the hair substrate were equally monitored.

5.3.2.2 Rotary drum composting (Phase 1)

Two identical stainless steel drums (Rowan Electronica, Italy) of a 2 litres capacity were used to simulate in-vessel composting process (Figure H2, Appendix H). Feedstock mixture

(1.5 kg) comprising of intact bovine hair, dry leaves, soil, and woodchips/saw dust at the proportions described in Section 5.3.2.1 were prepared and loaded into the two separate drums. These were considered as duplicate experiments. The initial moisture content of the material was adjusted and maintained at 55 ± 2% during the composting period, while the average pH of the two mixtures was 6.3 ± 1 at the start of the composting process. The carbon to nitrogen ratio determined on the dry samples of the starting material was 35 ± 1. Composting was performed under controlled conditions of moisture, and temperature maintained at a thermophilic range of 40 – 50°C for 120 days. The drums were periodically agitated to allow mixing and distribution of moisture within the matrix. During the composting period the temperature was regularly monitored by inserting a thermometer into the centre of the feedstock through the sampling pot and the temperature recorded. To maintain the moisture content in the drum within optimum required for high microbial activity, the feedstock was amended with sawdust as suggested by Haug (1980). Representative samples were periodically taken for chemical analysis, pH, moisture, and microscopy for changes in substrate structure characteristics. Chemical analysis was performed on samples dried overnight in oven at 105°C, ground and sieved through 1 mm sieve to obtain uniform particle size.

The average results and substrate characteristics from the two composting processes were taken as control and used to compare against the performance of the composting processes inoculated with the isolated microbial enzyme described below, Section 5.3.4.

5.3.2.3 Rotary drum composting (Phase 2: inoculated)

In order to study the effect of the isolated microbial enzyme in improving the decomposition of hair substrate, composting was performed in two identical drums containing the same proportion of feedstock mixture as described in Section 5.3.2.1. The initial moisture content of the feedstock was adjusted within the range of 55 ± 2% using Tris buffer of pH 9.0 and equilibrated for one hour. After equilibration the average final pH of the feedstock was 6.7 ± 2 and recorded as the initial pH. The two compost mixtures were each inoculated with a sufficient volume of the isolated microbial enzyme at the ratio of 1:17 (based on the weight of hair substrate, optimised in Chapter 4). The inoculation was carried out in three parts, as follows:

- At the 1st day of feedstock loading
- At the 60th day of composting
- At the 90th day of composting

5.3.3 Moisture content

Moisture content was determined gravimetrically by weight loss on drying. A measured mass of fresh compost sample was dried overnight in an oven at 105°C, and cooled in a desiccator to a constant weight as described by Allen (1974). Percentage moisture was calculated according to Equation 5.1, below.

$$\text{Moisture (\%)} = \frac{\text{Initial sample weight (g)} - \text{oven dry weight (g)}}{\text{Initial sample weight (g)}} \times 100$$

Equation 5.1

5.3.4 pH

pH was determined weekly in a suspension of a compost sample in deionised water (1:5 w/v), agitated for 30 minutes on a rotary shaker at ambient temperature and allowed to stand for 10 minutes as described by Allen (1974). Measurement was performed on a SevenMulti electrochemical pH meter (Mettler-Toledo Ltd, UK).

5.3.5 Organic carbon

Organic matter (OM) of the compost was determined by weight loss on ignition of oven dried samples in a muffle furnace at 560°C overnight and cooled in a desiccator to a constant weight. The percentage organic carbon was calculated from the organic matter (OM) according to the following Equations (Haug, 1980; Sutherland, 1998):

$$\text{Organic matter (\%)} = \frac{\text{Loss of weight due to ignition at 560°C (g)}}{\text{Oven dry weight at 105°C (g)}} \times 100$$

Equation 5.2

$$\text{Carbon (\%)} = \frac{\text{Organic matter (\%)}}{1.8}$$

Equation 5.3

5.3.6 Nitrogen

Percentage nitrogen was determined by the Total Kjeldahl Nitrogen method according to the procedure described by Vogel (1961).

5.3.7 Changes in humification

Humification level of the compost was determined by extracting 2 g of ground oven-dried samples, taken at different stages during composting, with 100 ml 0.1 M sodium hydroxide (NaOH) solution and 0.1 M sodium pyrophosphate ($N_4P_2O_7$) (Sigma Aldrich, UK) in an orbital shaker at 65°C for 48 hours, under nitrogen (Cavani, *et al.*, 2003). After the extraction the samples were centrifuged at 5000 r.p.m for 15 minutes and the supernatant filtered, this filtrate constituted the total extract (TE). Humic and fulvic acids were fractionated by acidifying 25 ml of the total extract (TE) with 6 M hydrochloric acid to pH 1.0, and allowed to stand overnight. The precipitated portion was collected by centrifugation at 5000 r.p.m for 20 minutes. The fraction precipitated and the supernatant represented humic acid (HA) and fulvic acid (FA), respectively. The acidification, precipitation and purification procedures were carried three times until no further precipitates were formed. The total extracted carbon (TEC) from the total extract (TE) and humified carbon in HA and FA was determined by the dichromate acid method of Walkley-Black, as described by Allison (1965). The full protocol is described in Appendix H1. All the determinations were carried out in duplicate and the mean values reported. The non-humified substance (NHS) was determined as the difference between TEC and (HA+FA). According to Ciavatta *et al.* (1988) the humification parameters, such as Degree of Humification (DH), Humification Rate (HR) and Humification Index (HI) were calculated according to the following Equations:

$$DH\ (\%) = \frac{(HA + FA)}{TEC} \times 100$$

Equation 5.4

$$HR\ (\%) = \frac{(HA + FA)}{C_{org}} \times 100$$

Equation 5.5

$$HI = \frac{TEC - (HA + FA)}{(HA + FA)}$$

Equation 5.6

Where TEC = Total extractable carbon and

C_{org} = organic carbon

5.3.8 Physical structure changes

The biological physical transformation of the substrate (hair) was progressively monitored by sampling at different stages (7, 15, 30, 60, 90 and 120 days) during the composting process and examined microscopically to ascertain the extent of breakdown. Samples were prepared for histological examination and examined using scanning electron microscope (Hitachi, S-3000N Japan), as described in Section 2.2.2.1.1.1.

5.3.9 Statistical analysis

All the results are presented as the mean (mean ± confidence interval) of duplicates and were analysed for significance difference using one way ANOVA, as described in Section 2.3. The probability level used for statistical significance was $p \leq 0.05$.

5.4 Results and discussion

5.4.1 Laboratory-scale composting trial

The operational process parameters evaluated during the 40 days of laboratory composting trial were pH, moisture, and temperature. The initial objective was to determine if degradation of hair could be achieved under a static composting environment by emulating those conditions at which microbial degradation was observed to occur during the laboratory incubation study. The average temperature and moisture profiles of the feedstock observed during the experimental period are shown in Figures 5.3 and 5.4, respectively. The average feedstock temperatures presented were the actual thermometer readings recorded, and compared against the regulated incubator temperature (control temperature). As shown in Figure 5.3, the initial temperature recorded was 40°C, but the average temperature within the feedstock dropped to 37°C after one week of incubation.

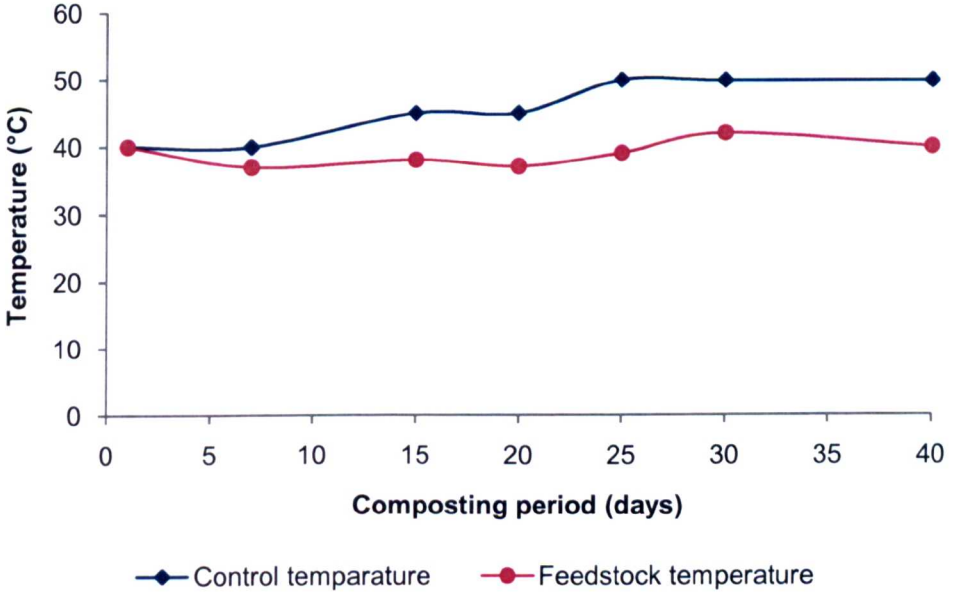

Figure 5.3: Temperature profiles of the laboratory-scale static composting trial.

Subsequent adjustments of the incubator control temperature to 45 and 50°C at 2 weeks and 30 days of composting, respectively, only caused the temperature of the feedstock rise to an average of 39 ± 1°C. There was variation of temperature within the depth of the pile. Temperature at the bottom-end of the feedstock was always lower (approximately ± 2°C) than the middle part. Temperature is an important process parameter to achieve composting efficiency, since it affects both the biological reaction rates and the dynamics of microbial

population (Fogarty and Tuovinen, 1991). It was evident from the temperature profiles that the expected optimum range of temperature of 45 – 50°C was not being achieved.

There are two possible reasons that could have been responsible for the failure of the compost to achieve the required temperature. First, although the composting bin was designed to minimise heat loss, it may be possible that it was not well insulated. Secondly there was improper distribution of moisture, which was often characterised by accumulation of condensed water in the middle of the feedstock. The accumulation of water may have had a cooling effect and inadequate heat distribution within the entire feedstock. As a consequence of condensation moisture content tended to increase in localised regions within the feedstock and was often high (> 57%), as shown in Figure 5.4. Although the initial moisture content was adjusted to 54 ± 2%, there was an increase in moisture content to an average of 65 ± 3% in the first week, rising to a maximum of 76 ± 2% after 20 days of incubation. Generally, moisture content in the range of 50 to 60% is considered optimal for high microbial activity (Liang *et al.*, 2003; Golueke, 1972).

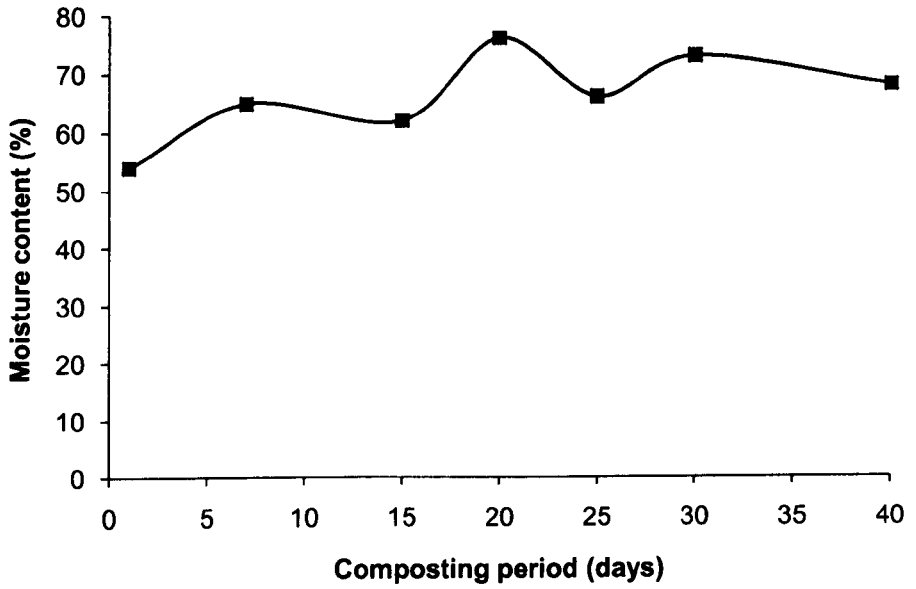

Figure 5.4: Profiles of the average moisture content of the feedstock during the laboratory-scale static composting trial.

Despite efforts to regulate the moisture content with the addition of sawdust, it was difficult to control under the experimental conditions. Subsequently, the high localised moisture content appeared to affect the process as a strong odour was experienced, which suggested that there was an inadequate supply of oxygen within the feedstock and anaerobic

decomposition had been initiated. Attempts to improve the process by regular turning of the feedstock did not improve the situation.

The pH of the feedstock during the incubation ranged from 6.5 to 7.6. The overall pH of the process was 6.9 ± 0.5, which was within the favourable range for microbial activity. Due to the inability to achieve an optimum operating temperature, poor aeration and difficulties in regulating the moisture content of the feedstock, the composting process was terminated at 40 days. By comparing the structural changes of the substrate (Figure 5.6) with the control sample (5.5) it can be clearly seen that no degradation had taken place after 40 days incubation. The surface of the substrate was mainly covered with mycelial-like (see arrow, Figure 5.6) structures suggesting fungal growth. The results indicate that under the designed static composting trial conditions the decomposition of the substrate was poor.

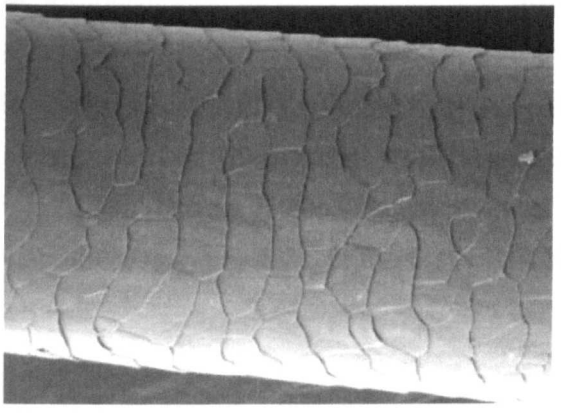

Figure 5.5: SEM image of an intact hair shaft (mag. x1.0K, 5.0kV).

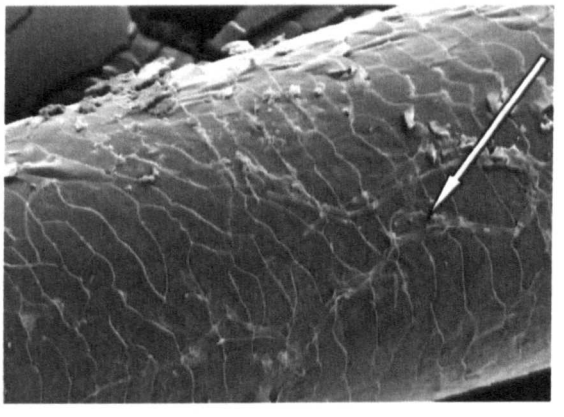

Figure 5.6: SEM image of hair shaft illustrating mycelial growth following laboratory static composting for 40 days (mag. x1.0K, 5.0kV).

5.4.2 Rotary drum composting

5.4.2.1 Controlled in-vessel composting: Phase 1

Phase 1 of the experiment involved composting the feedstock mixture without the inoculant under controlled environmental process conditions of temperature, moisture and pH. Particular attention was to maintain optimum parameters suitable for high microbial activity necessary for the decomposition of highly resistant substrates. The keratinolytic micro-organism with the ability to decompose hair, reported previously in Chapter 4 was found to be active in the temperature range of 40 – 50°C. In a composting environment this refers to thermophilic range (> 35 °C) (Insam and De Bertoldi, 2007). Therefore, the drum composting process was carried out under controlled temperature range of 40 – 50°C, favourable for the

proliferation of the micro-organism. The capability of thermophilic bacteria to decompose highly resistant animal proteins such as prions, elastin and collagen have been studied (Suzuki *et al.*, 2006). The main function of the composting unit is to provide optimal environmental conditions for the microbial population to carry out their metabolic activities (Finstein, 1980). As such, in this study, working with small quantities of feedstock materials and the choice of rotating drum for the process, was to allow a higher degree of control of the process parameters as suggested by Schulze (1962), Mason and Milke (2005).

5.4.2.1.1 Temperature

Temperature has been identified as a key factor, and perhaps the most important parameter that affects microbial metabolism within a composting environment (Finstein and Morris, 1975; Fogarty and Tuovinen, 1991; Stentiford, 1996). Whilst microbial diversity is maximised at temperatures ranging from 35 – 40°C, a higher rate of microbial biodegradation is maximised between 45 – 55°C. On the other hand, temperatures higher than 55°C maximises sanitisation (Finstein *et al.*, 1983; Stentiford, 1996). Figure 5.7 shows the profile of a controlled thermostat drum temperature and the measured temperature within the feedstock. The monitored temperature of the feedstock shows that the initial temperature was 40°C, rising to 43°C in the first week of composting.

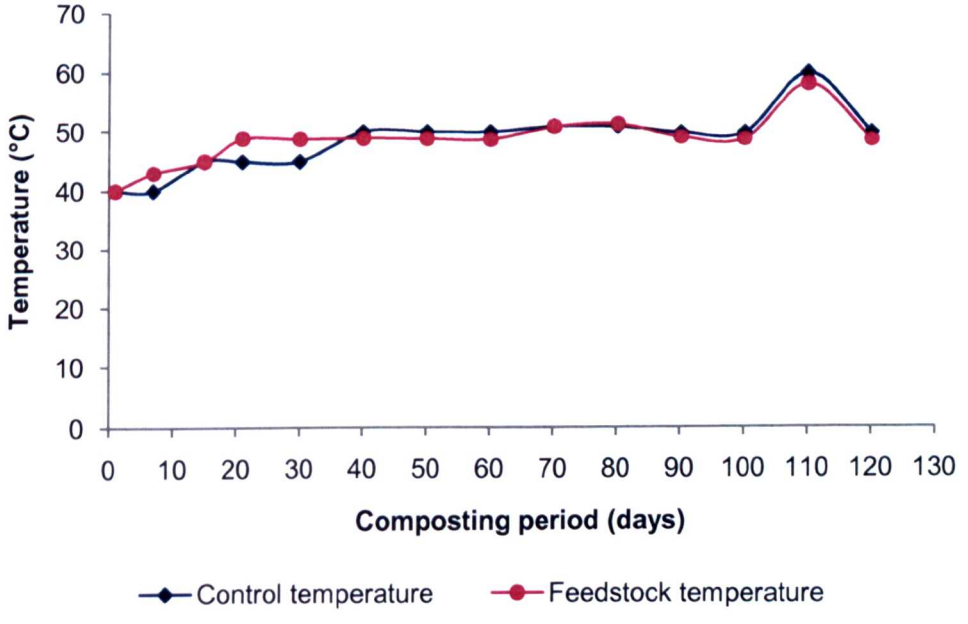

Figure 5.7: Thermophilic temperature profile (49 ± 2°C) during rotary drum in-vessel controlled composting for 120 days: Phase 1 composting.

The control temperature was increased to 45°C in the second week of the composting process, and maintained at that temperature for two (2) additional weeks. During this period the monitored temperature within the feedstock was 47 ± 2, thus providing indications that the intended optimum temperature was being achieved. The drum temperature was further increased and controlled at 50 ± 1°C for 60 days to encourage the proliferation of thermophilic bacteria, specifically those with the capability of metabolising keratin. During this period the monitored temperature within the feedstock was 49 ± 2°C.

Although the microbial community in a composting environment is diverse (Finstein and Morris, 1975; Strom, 1985a), on the basis of the micro-organism identified in Chapter 4, the specific class of bacteria that was of interest was the *Bacillus* species. These species are known to degrade a wide range of organic materials including keratin, and have been used in biotechnological processes such as conversion of feathers into feed ingredients, and for unhairing and bating in the tanning industry (Alexander and Walter, 2005; Dalev, 1994; Hameed, *et al.*, 1996; Lin *et al.*, 1992; Raju *et al.*, 1996; William *et al.*, 1991). They have a broad range of growth temperatures, with different strains exhibiting optimum stability temperatures from 40 – 80°C (Gibson and Ruth, 1974; Horikoshi, 1996; Strom, 1985b). In this study, the thermophilic temperature was maintained for long periods of time in order to maximise and sustain high metabolic activity of the bacteria during the active composting process. The strategy was consistent with the literature reviews, where it is reported that the rate of microbial bio-oxidation is higher at the thermophilic temperature range of 45 to 55°C, whereas higher temperatures slows down the decomposition of organic materials due to inhibition of microbial growth (Stentiford, 1996, 1993). In this study, the controlled temperature regime included a pasteurisation process, observed at a peak temperature of 58°C, for a constant period of five days as recommended by the regulatory requirements for the destruction of pathogens (USEPA, 1979). Pasteurisation was followed by a gradual decrease of temperature up to 47 ± 1°C as a conditioning process for the compost (Rai and Ahlawat, 2002).

5.4.2.1.2 Moisture

The successful decomposition of an organic material depends on the fulfilment of parameters favourable to sustain metabolic and cellular functions of micro-organisms in the biological system (Diaz and Savage, 2007; Haug, 1980). Moisture content during composting is an important environmental factor as it provides a medium for the transportation of dissolved nutrients and transfer of waste products, thus enabling the micro-organisms to perform their metabolic and physiological activities. Moisture influences both

the structural and thermal properties of the material, and although the amount varies depending on the material, the optimum initial moisture content recommended for efficient composting ranges between 55 – 65% (Diaz and Savage, 2007; Haug, 1980; Stentiford, 1996; Vallini *et al.*, 2002). On the basis of a wide range of research results, the appropriate moisture content for operating a composting process should be in the range of 40 – 60% (Stentiford, 1996; Tiquia *et al.*, 1996). Excessive moisture inhibits aerobic metabolism due to limitations on oxygen diffusion, and presents the risk of anaerobic conditions as well as emission of foul odours. On the other hand, biodegradation is slowed in the compost mixtures with below 40% moisture content (Fogarty and Tuovinen, 1991; Poincelot, 1974; Stentiford, 1996; Vallini *et al.*, 2002).

The profile of the moisture content during the controlled composting experiment is shown in Figure 5.8. The initial moisture content of the feedstock was 55%, considered optimum for microbial activity. In the first week there was a slight increase in the moisture content to about 57%. This was attributed to the accumulation of condensed water as a result of the occasional cooling effect in the thermostatic drum. As the composting progressed, variation in the moisture content was encountered, with occasional drying and minimal increases in the next 2 weeks. Subsequently, although there was a declining trend, the process maintained moisture content of 55 ± 1% for about 60 days.

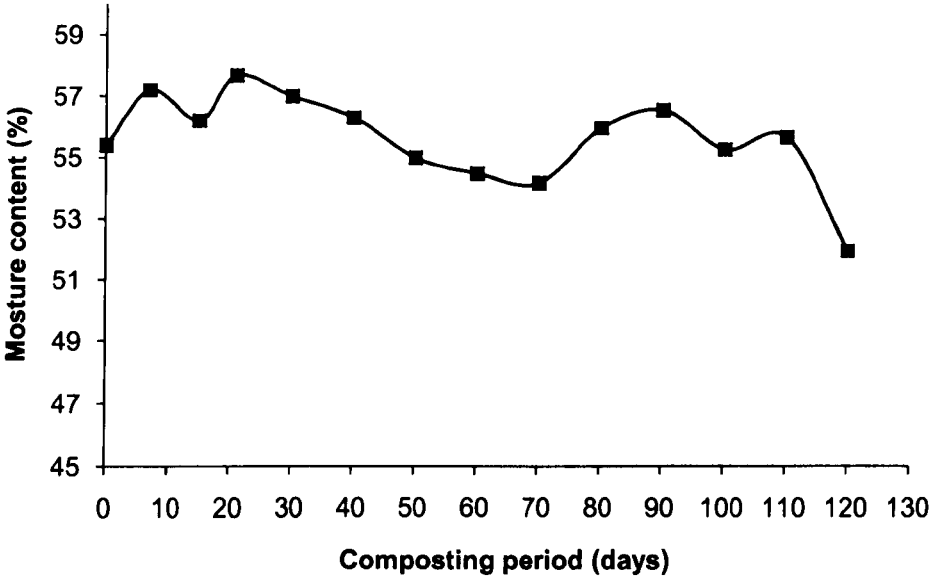

Figure 5.8: Profile of moisture content (55 ± 2°C) during rotary drum in-vessel controlled composting for 120 days: Phase 1 composting.

The period (between 30 – 90 days) which was characterised by declining moisture content, was considered to be the most active period of microbial activity as it was also the period when a temperature of 51°C was obtained within the feedstock. Thus, it was an indication the control process was working well. The justification of this assumption may be explained by the fact that loss of water as result of evaporation as composting progresses, is considered a major part of the process, and evolution of temperature is a reflection of microbial activity (Finstein *et al.*, 1986; Stentiford, 1996). The average moisture content maintained during the entire period was 55 ± 2%. The material sampled at the end of the experiment had a moisture content of 51 ± 1%.

5.4.2.1.3 pH

The optimal range of pH for operating a composting process should be within the levels tolerable to micro-organisms. This is widely accepted to be in the range of pH 5.5 – 8.0. Whereas bacteria generally prefer a near neutral range of pH 6.0 – 7.5, fungi grow well in acidic conditions (Diaz and Savage; 2007; Fogarty and Tuovinen, 1991; Golueke, 1972; Haug, 1980). At thermophilic temperatures composting proceeds more efficiently within the pH range 6.5 – 8.5 (Jeris and Regan, 1973a; Liao *et al.*, 1997). However, in terms of operational parameters, precaution has to be taken noting that alkaline conditions of pH above 8.5 at thermophilic temperature favour the loss of nitrogen through volatilisation of ammonia. This may contribute to the unpleasant odour emissions from the composting matrix (Diaz and Savage, 2007; Vallini *et al.*, 2002; Stentiford and Pereira, 1985).

Figure 5.9 represents the average pH values monitored in the replicate mixtures during composting. The initial average pH of the feedstock mixtures at loading was 6.3, which was within the range suitable for microbial activity. However, there was a gradual increase in the pH to about 6.8 after the first 2 weeks. There was no significant variation between the mixtures in the duplicate drums, and after one month the process attained self-buffering at pH 7.0 for almost two months before rising again to about 7.7. The rise in the pH could be attributed to the proteolytic process as a consequence of microbial metabolism, which may have led to a possible volatilisation of ammonia during the decomposition of organic materials (Chefetz *et al.*, 1998; De Nobili and Petrussi, 1988; Diaz and Savage, 2007).

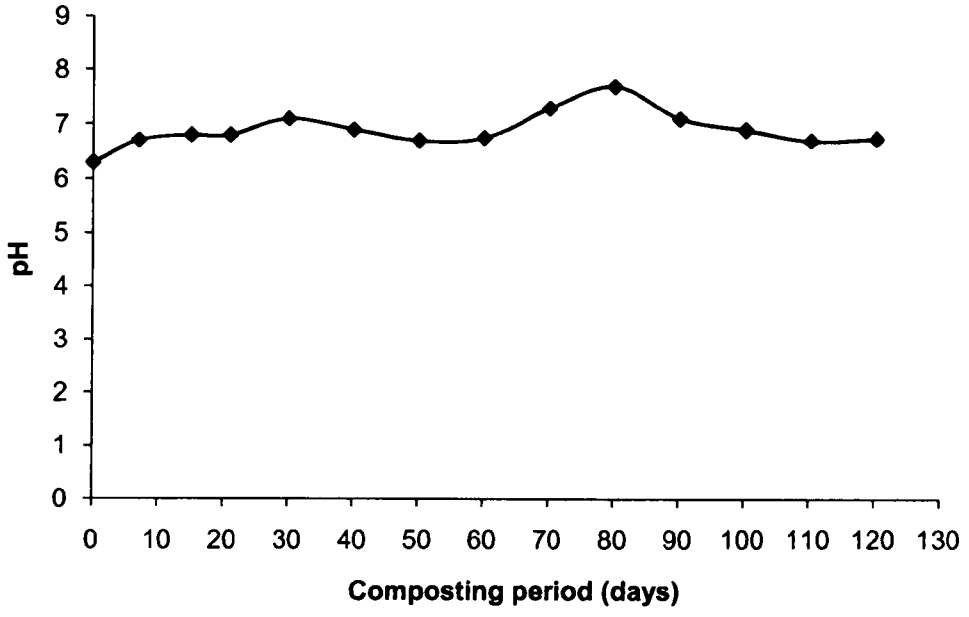

Figure 5.9: pH profile (7.0 ± 0.2) during rotary drum in-vessel controlled composting for 120 days: Phase 1 composting.

The slight alkaline conditions lasted for ten days, after which the pH gradually dropped to a final average of 6.7 ± 0.4 at the termination of the experiment. This was consistent with the literature which explains that the decrease in pH to a near neutral pH is due to the increased buffering capacity as result of humus formation (Poincelot, 1974). The overall pattern of the pH monitored during the composting process was comparable with other research observations (Khalil *et al.*, 2001; Pearson *et al.*, 2004).

5.4.2.1.4 Aeration

Composting is concerned primarily with the biological oxidation of the organic waste material through microbial metabolism. Consequently, it is desirable that the process be carried out under aerobic conditions. Thus the availability of oxygen is one of the principal factors that control the process (Haug, 1980; Fogarty and Tuovinen, 1991; Stentiford, 1996). In the absence or limitation of supply of oxygen, the rate of decomposition is reduced, with fermentation and anaerobic conditions developing. In contrast, excessive aeration limits microbial activity as a result of the associated drying and cooling. This is to say, aeration also influences thermal properties and moisture content of the composting process (Finstein *et al.*, 1983; Fogarty and Tuovinen, 1991; Stentiford, 1996; Vallini *et al.*, 2002). A number of commonly used aeration methods include natural, passive, forced aeration, and agitation (Diaz *et al.*, 2007; Haug, 1980; Sartaj *et al.*, 1995; Stentiford, 1996). In this experiment, the two drums were periodically agitated to ensure sufficient and uniform aeration within the

compost matrix. Oxygen was supplied through an external vent (Figure H2, Appendix H). The mechanical agitation further allowed adequate mixing and distribution of moisture thus maintaining optimum conditions necessary for the microbial biological processes. Clamped materials were manually mixed to maintain porosity within the matrix.

5.4.2.1.5 Changes in carbon and nitrogen (C/N)

Carbon and nitrogen are essential macronutrients needed by the micro-organisms for their metabolic and cellular functions during the oxidative decomposition process. The nutrients must not only be chemically available but also physically available in the correct proportion to be accessible by the particular micro-organism (Diaz and Savage, 2007). The substrate is the source of the essential nutrients. The optimum proportion of the starting ratio of carbon to nitrogen ranges between 20:1 and 35:1 (Diaz and Savage, 2007; Haug, 1980). In the course of carrying out metabolic processes under aerobic conditions, micro-organisms transform the organic matter (substrate) essentially into carbon dioxide, water, mineral ions and heat energy. The transformation of the organic carbon into inorganic carbon (CO_2) is referred to as mineralisation (Calmon *et al.*, 1999; Diaz and Savage, 2007; Grima *et al.*, 2000; Haug, 1980).

Table 5.1 shows the proportion of carbon and nitrogen (C/N) in the starting material and the changes that occurred as composting progressed. The ratio of carbon to nitrogen present in the starting material was about 35:1, which was within the optimum range recommended to sustain microbial metabolism.

Table 5.1: Changes in organic carbon and total nitrogen during phase 1 uninoculated rotary drum in-vessel composting process: (percentages are mean values based on oven dried samples at 105°C).

Composting day	Organic carbon (%)*	Nitrogen (%)*	C/N ratio
0	56 ± 2.0	1.62 ± 0.2	35 ± 2
15	50 ± 4.0	1.50 ± 0.2	33 ± 2
30	53 ± 1.0	1.73 ± 0.04	31 ± 1
40	51 ± 3.0	1.64 ± 0.11	31 ± 2
60	54 ± 1.0	1.80 ± 0.06	30 ± 1
80	52 ± 2.0	1.70 ± 0.1	31 ± 3
90	51 ± 2.0	1.58 ± 0.05	32 ± 1
100	48 ± 3.0	1.54 ± 0.1	31 ± 2
120	41 ± 2.0	1.40 ± 0.03	29 ± 1

Samples were taken from 2 different experimental drums. *Mean of four duplicates ± confidence interval.

After two weeks of composting there was a slight decrease in the carbon to nitrogen ratios, 33:1. This may be explained by the fact that during the composting process organic carbon is lost as CO_2, which progressively reduces the carbon to nitrogen ratio (Insam and de Bertoldi, 2007; Finstein *et al.*, 1986). There was a variable fluctuation in percentage nitrogen, with increases observed during the first month of composting, 1.73 ± 0.04%, and the second month, 1.80 ± 0.06, followed by a decline towards the end of the composting experiment. Increase in nitrogen during composting have been reported in other research findings, and may depend on the substrate being composted (Bernal *et al.*, 1996; Hansen *et al.*, 1991). On the other hand the decreases in the percent nitrogen during the process may suggest some losses through ammonia volatilisation, which may occur during the composting process (Insam and de Bertoldi, 2007; Finstein and Morris, 1975).

Although the progressive change in the ratio of carbon to nitrogen was not significant, the general observation was that the proportion of carbon to nitrogen was on the decline as composting progressed. The decline was attributed to the biological process of mineralisation, a microbial metabolic decomposition process leading to the loss of the organic carbon as CO_2 as observed by Finstein *et al.* (1986). Besides the mineralisation process, the trends in the decrease in the C/N ratios have been used as a criterion to suggest progressive stabilisation of the compost (Chefetz *et al.*, 1996; Harada *et al.*, 1981). A carbon to nitrogen ratio of less than 20 is sometimes used as an indicator of stabilised compost. However, C/N ratios of 19 to 31 have been reported in stabilised composts of different materials (Inoko, *et al.*, 1979). Due to such variability it has been suggested that, C/N ratio, should not be used alone as an absolute indicator of compost stability. In addition, the actual C/N content of the final compost is dependent on the C/N ratio of the starting material and the extent of biodegradation (Crawford, 1983; Hirai *et al.*, 1983; Jimenez and Garcia, 1989).

The average ratio of carbon to nitrogen of the compost sampled at the termination of the experiment was 29 ± 1. This was a high value compared to the suggested range, (<20), that represent maturity. However, in addition to the suggestion that the actual C/N ratio of the final compost depends on the proportion present in the starting material, in this study, the high C/N ratio may also suggest that decomposition of the substrate was slow, probably due to the complex structure of the keratinous substrate. It has also been suggested that the value of C/N ratio may be above 20 in mature compost because part of the organic carbon may be in the form of compounds more resistant to biodegradation, particularly lignin, and thus not readily available to micro-organisms (Jeris and Regan, 1973b; Regan and Jeris, 1970). Lignin is among the naturally occurring polymers which is highly resistant to

biodegradation due to its highly crosslinked structure. It is available as a major component of plant materials (Crawford, 1981).

5.4.2.2 Structural changes of the substrate (hair) during composting: (Phase 1)

Composting is biodegradation of a mixture of substrates carried out by complex microbial populations under aerobic conditions. During the composting process the substrate may undergo physical and biological changes, accomplished by enzymatic processes and the predominant indigenous microbes which are specific to the material being composted (Haug, 1980; Insam and De Bertoldi, 2007). The decomposition of keratin is mediated by a selective group of enzymes collectively referred to as keratinases. Keratinases are secreted extracellularly by keratinolytic thermophilic bacteria that belong to the family of serine-proteases (Lin *et al.*, 1992; Mancziger *et al.*, 2003; Onifade *et al.*, 1998). In the previous chapter (Chapter 4), it was demonstrated that the keratinolytic activity of the keratinases could be induced by incubating hair with soil under controlled laboratory conditions. The characteristics of the microbial enzymes, therefore, led to the assumption that the micro-organisms can be involved in the biodegradation of hair under the thermophilic composting environment.

The results from the degradation of hair under the controlled composting environment are represented in Figures 5.10 – 5.15. Figures illustrate changes manifested at different periods during the composting process. The initial effects of biodegradation were characterised by isolated surface erosions of the cuticle layer, observed in the first week of composting (Figure 5.10). This was typical of keratinolytic enzymes as previously observed in Chapter 3 and 4. After 15 days of composting the substrate retained its physical integrity but it was evident that besides enzymatic degradation, mechanical decomposition was also involved in facilitating the breakdown (Figure 5.11).

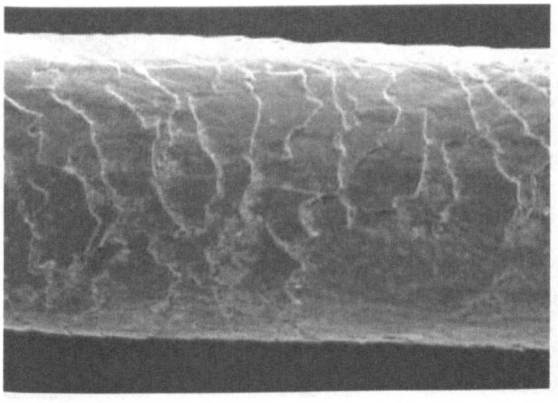

Figure 5.10: SEM image of hair shaft illustrating damage after 7 days of uninoculated composting (mag. x1.0K, 5.0kV).

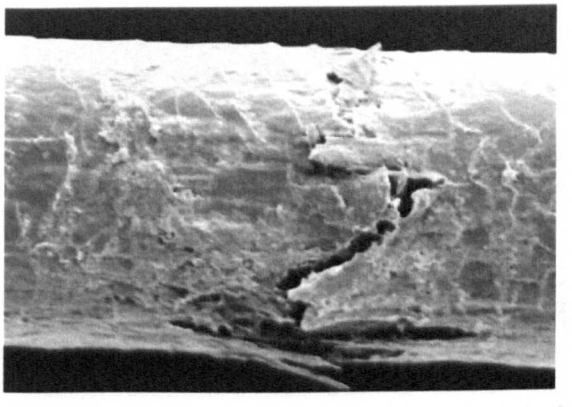

Figure 5.11: SEM image of hair shaft illustrating damage following 15 days of uninoculated composting (mag. x1.0K, 5.0kV).

Microbial decomposition in a composting environment is known to largely depend on the appropriate environmental conditions of moisture content, temperature, oxygen and on the availability of nutritive substances, particularly carbon and nitrogen (Diaz and Savage, 2007; Stentiford, 1996). The following microscopy images (Figures 5.12 – 5.15) of hair undergoing degradation provides the evidence that the micro-organisms were able to metabolise hair. Thus, it demonstrates that during the controlled composting experiment, optimal conditions that could stimulate and sustain the proliferation of the specific beneficial micro-organisms were satisfied. Figure 5.12 shows the advanced keratinolytic delamination of the cuticle and mechanical splitting of the substrate after 30 days of composting. The microscopy image also demonstrates that although biodegradation was progressive the highly keratinised components were slowly degraded.

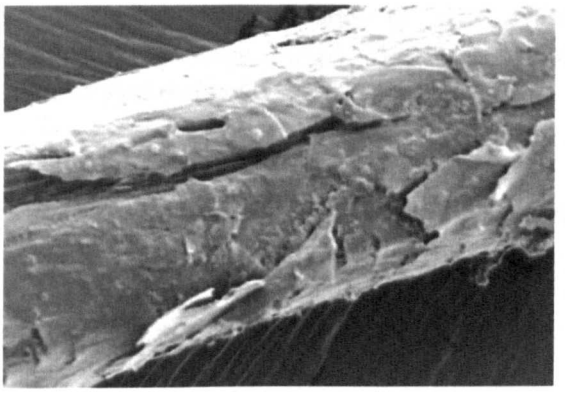

Figure 5.12: SEM image of a hair shaft illustrating damage after 30 days of uninoculated composting (mag. x1.0K, 5.0kV).

Figures 5.13 and 5.14 illustrate the physical characteristics of the substrate after 60 and 90 days, respectively. At 60 days most of the surface cuticle had fallen, although the highly keratinised cortical fibres were still intact. Figure 5.14 illustrates the disintegration and fibrillation of the substrate.

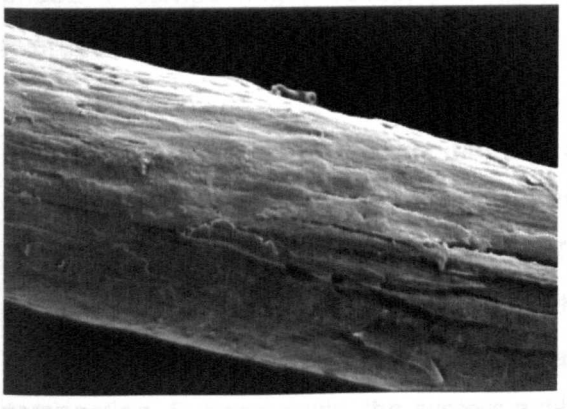

Figure 5.13: SEM image of a hair shaft illustrating damage after 60 days of uninoculated composting (mag. x1.0K, 5.0kV).

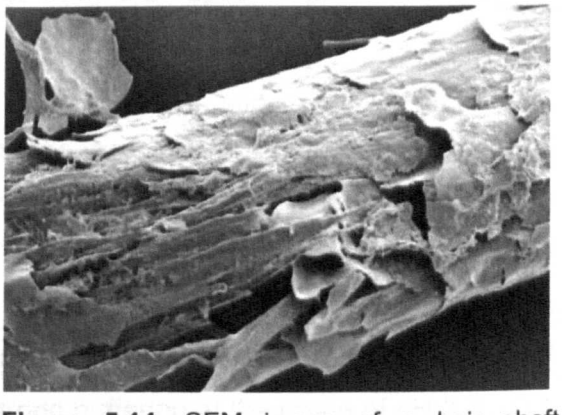

Figure 5.14: SEM image of a hair shaft illustrating damage after 90 days of uninoculated composting (mag. x1.0K, 5.0kV).

At the conclusion of the experiment, the substrate had undergone major disintegration leaving cuticular fragments (Figure 5.15) which were more resistant and did not biodegrade/decompose within the experimental period.

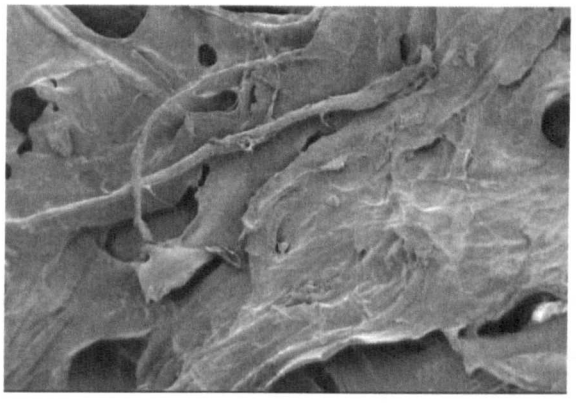

Figure 5.15: SEM image of hair shaft illustrating damage after 120 days of uninoculated composting (mag. x1.0K, 5.0kV).

5.4.2.3 Controlled in-vessel composting: Phase 2 (inoculation with microbial keratinolytic crude enzyme)

5.4.2.3.1 Temperature, moisture and pH

The operational process parameters of temperature, moisture and pH, followed similar control and monitoring procedures adapted in the phase 1 drum composting (un-inoculated process, Sections 5.4.2.1.1, 5.4.2.1.2 and 5.4.2.1.3). The control temperature during the active composting process was kept at the optimum thermophilic range of 50 ± 1°C, and included a pasteurisation at a peak temperature of 59 ± 1°C, that lasted for five days. The moisture content in the duplicate drums was kept within the recommended range 55 – 58% during the composting process, which corresponded to the moisture conditions of the initial phase 1 composting process. The final compost from the replicate processes had an average moisture content of 48 ± 1%.

Figure 5.16 represents the pH profiles of both phase 1 (un-inoculated) and phase 2 (inoculated) composting processes. The initial pH of the inoculated raw feedstock mixture prepared for phase 2 composting was 6.7 ± 2. The start of composting was marked by a gradual rise in the pH, reaching 6.9 in the first week. However, it showed a significant increase in the following two weeks, reaching a peak value of 8.3 on the 21st day of composting. Subsequently, the system exhibited strong buffering at the range of pH 8.2 ± 0.1 for thirty days followed by an increase to a maximum of pH 8.4, which was recorded

between the 60th and 70th day of composting. The pH then proceeded to decrease gradually to a final average of 7.7 ± 0.1 at the termination of the experiment. The overall average operational pH during the course of the composting process was 8.0 ± 0.2. Comparatively, the pattern of the pH during the phase 2 composting process was found to vary significantly with the pH profiles of phase 1 process. The relatively high pH may have been influenced by the inoculant or volatilisation of the products of decomposition following increased enzymatic degradation of the substrate.

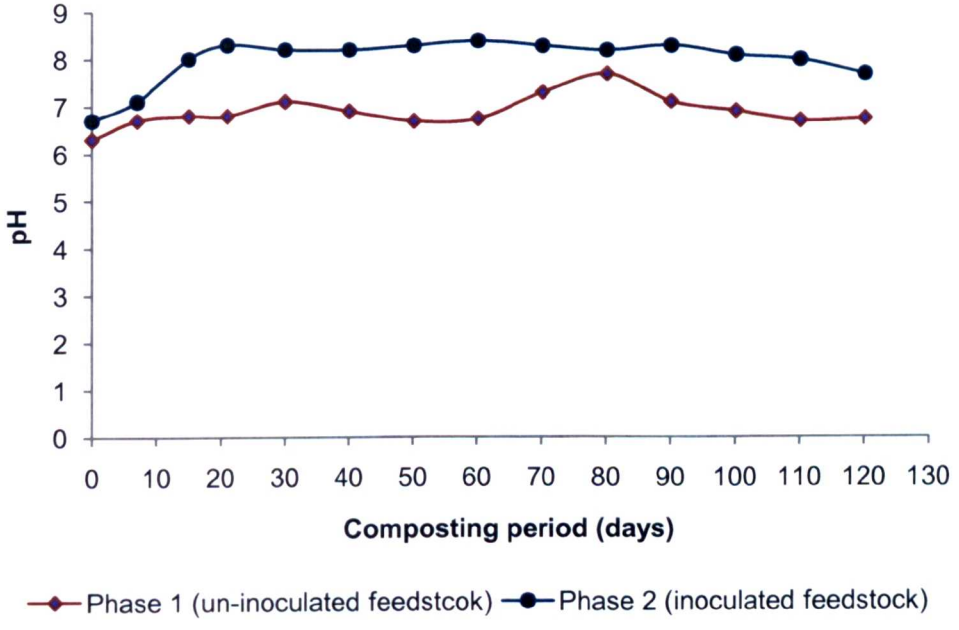

Figure 5.16: Comparative pH profiles of the Phase 1 (pH 7.0 ± 0.2) and Phase 2 (pH 8.0 ± 0.2) during rotary drum in-vessel controlled composting.

In the previous chapter (Chapter 4), it was established that the isolated microbial crude enzyme was active in the pH range 7.5 – 10 with an optimum activity at pH 9.0. While it was noted that the course of the pH within the composting natural environment was below the pH of optimum activity it was equally important to avoid high pH values which would have favoured loss of nitrogen through volatilisation of ammonia (Pagans *et al.*, 2006; Stentiford and Pereira, 1985, Vallini *et al.*, 2002). However, the observed pH profiles were within the range of the microbial enzyme activity, and at a favourable range when composting is considered to proceed efficiently at thermophilic temperatures (Jeris and Regan, 1973a; Liao *et al.*, 1997). As already explained in Section 5.4.2.1.3, the subsequent rise in the pH to a maximum 8.4 may have been as a result of the proteolytic enzyme decomposition of the organic substrate leading to a possible volatilisation of ammonia (Chefetz *et al.*, 1998; De Nobili and Petrussi, 1988).

5.4.2.3.2 Changes in carbon and nitrogen (Phase 2)

Results from carbon and nitrogen measurements during the phase 2 inoculated composting experiments are shown in Table 5.2. The compost product had a carbon content of 37% and a total nitrogen content of 1.5% at the termination of the experiment. This represented a reduction of 36% in carbon content and 11% total nitrogen. A significant difference ($p < 0.05$) in the percentage of organic carbon mineralised (36%) was observed in composts with inoculant when compared to composts without inoculant (27%). However, there was no significant difference ($p > 0.05$) in the total nitrogen content lost during the inoculated composting process (11%) compared with phase 1 uninoculated process (14%). The higher percentage of mineralised carbon during the inoculated composting process may be attributed to the increased bioconversion efficiency of the isolated microbial enzyme, present in the inoculant, leading to an increase in CO_2 evolution as the organic substrate was decomposed and transformed into new products. The C/N ratio in the inoculated composting process was 25:1, which was 14% lower than the phase 1 uninoculated composting process. This suggests improved stabilisation of the compost when compared to phase 1 composting process.

Table 5.2: Changes in organic carbon and nitrogen during Phase 2 inoculated composting process (percentages are mean values based on oven dried samples at 105°C).

Composting period (days)	Organic carbon (%)*	Nitrogen (%)*	C/N ratio
0	58 ± 2.0	1.66 ± 0.1	35 ± 2.0
15	54 ± 2.0	1.60 ± 0.03	34 ± 0.7
30	49 ± 3.0	1.65 ± 0.05	30 ± 1.0
40	52 ± 1.0	1.61 ± 0.1	32 ± 0.4
60	48 ± 3.0	1.70 ± 0.03	28 ± 1.2
80	50 ± 0.5	1.59 ± 0.06	31 ± 2.0
90	46 ± 2.0	1.64 ± 0.02	28 ± 2.0
100	43 ± 1.0	1.56 ± 0.05	28 ± 1.3
120	37 ± 1.0	1.47 ± 0.03	25 ± 2.0

Samples were taken from 2 different experimental drums. *Mean of four duplicates ± confidence interval.

5.4.2.4 Structural changes of the substrate (hair) during composting (Phase 2)

The main structural constituent of hair is keratin but it also contains a fraction of other non-keratinous and non-proteinaceous materials (Feughelman, 2002; Fraser *et al.*, 1972). Under natural conditions the biodegradation of keratinous substrates is difficult due to their macromolecular properties and highly crosslinked structural characteristics. Therefore, hair is more resistant to microbial degradation compared to other animal structural proteins such as collagen and elastin. The capability of particular micro-organisms to assimilate keratinous substrates depends on their ability to produce the enzymes needed to undertake the degradation. Although it has been demonstrated in this research that a specific group of micro-organisms naturally present in the environment are capable of carrying out the degradation process, inoculation with keratinolytic enzymes is a strategy that could potentially enhance the biodegradation process and improve the properties of the final product. The application of inoculants to enhance composting efficiency of organic solid wastes has been studied by others (Kaosol and wandee, 2009; Shin *et al.*, 1999; Xi *et al.*, 2005; Xi and Liu, 2002; Yadav *et al.*, 1982). Nakasaki *et al.* (1994) have reportedly used thermophilic bacterium to accelerate the decomposition of organic matter during composting. The catalytic diversity of microbial keratinases have made their application as biocatalysts, in bioprocesses that convert keratinous waste into value-added products such as feeds and fertilisers, increasingly attractive (Brandelli, 2008; Brandelli *et al.*, 2010; Grazziotin *et al.*, 2006; Gupta and Ramnani, 2006; Haddar *et al.*, 2009; Ichida *et al.*, 2001; Onifade *et al.*, 1998; Tiquia *et al.*, 2005).

In Chapter 4 of this thesis, it was found that due to its complex and rigid structure, the enhancement of hair degradation required the presence of a more complex mixture of enzymes working through a synergistic action. The results of the effects of incorporating the isolated microbial enzyme as an inoculant during the composting of hair are illustrated in Figures 5.17 – 5.22. The microscopy (SEM) observation of surface morphology showed that there was an improvement in surface degradation (Figure 5.17) in the first week of composting compared to the surface morphology of the specimen from uninoculated composting process examined for the same period (Figure 5.10). Figure 5.18 shows the structure of hair after two weeks (15 days) of composting. Besides increased degradation of the surface, degradation of the inner structural components and disintegration of the substrate was evident at this early stage of composting (Figure 5.18).

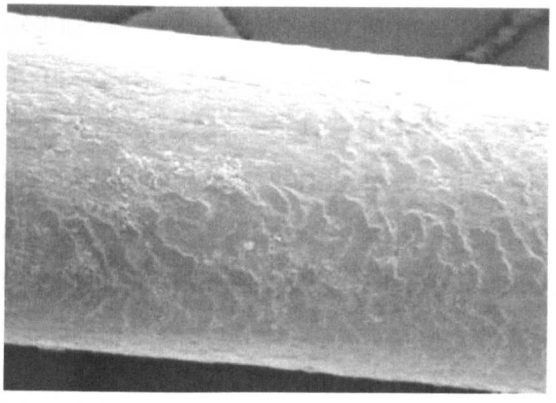

Figure 5.17: SEM image of a hair shaft illustrating damage after 7 days of inoculated composting (mag. x1.0K, 5.0kV).

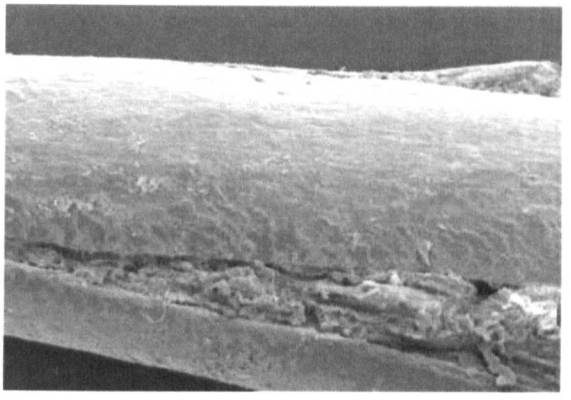

Figure 5.18: SEM image of a hair shaft illustrating damage after 15 days of inoculated composting (mag. x1.0K, 5.0kV).

Details of the structural changes of the substrate after 30 and 60 days of composting in the presence of the microbial enzyme inoculant are shown in Figures 5.19 and 5.20. The influence of the inoculant in the decomposition of the substrate was illustrated by the enhanced defragmentation of the structures, shown in the SEM images (Figures 5.19 & 5.20). The images show the degradation of the inner non-keratinous components and loss of structural integrity. In comparison to the uninoculated samples examined for the same period (Figures 5.12 & 5.13) there was evidence of improved structural modification in the inoculated composting process.

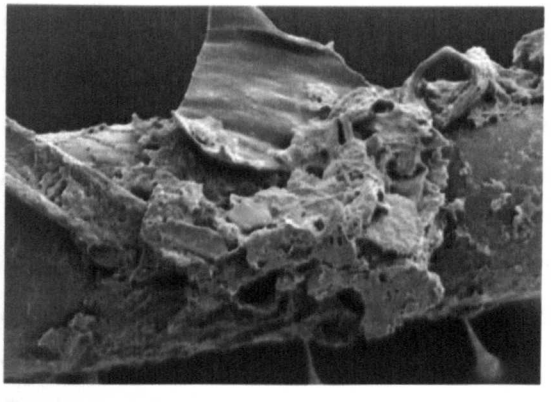

Figure 5.19: SEM image of hair shaft illustrating damage after 30 days of inoculated composting (mag. x1.0K, 5.0kV).

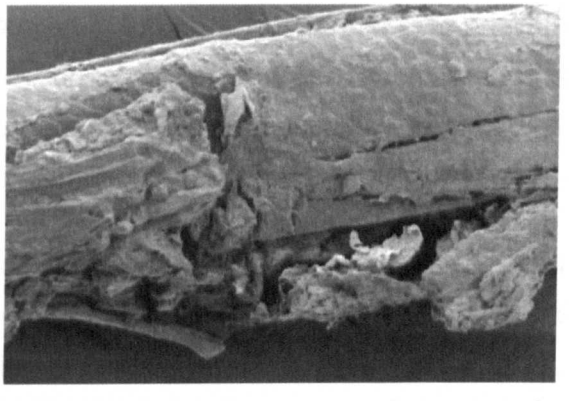

Figure 5.20: SEM image of hair shaft illustrating damage after 60 days of inoculated composting (mag. x1.0K, 5.0kV).

Additional evidence of enhanced degradation was observed by extensive fibrillation of the structures of specimen samples examined after 90 days of composting. This is illustrated in Figure 5.21. The splitting and fibrillation of the substrate demonstrates degradation of intercellular materials and non-keratinous components, believed to have been as a result of the combined action of the isolated microbial enzyme as an inoculant during composting. The combined action of the inoculant (the added microbial enzyme) was as a keratinase,

acting on keratinised hair components, and as protease, acting on non-keratinised hair components. Figure 5.22 illustrates the remaining structural components, the undegraded cuticle fragments, after 120 days of composting. Although the efficiency of the extent of biodegradation with the isolated microbial enzyme appeared to improve at different stages as composting progressed, in comparison, microscopic visualisation of the specimen samples from uninoculated and inoculated composting processes demonstrated fragmented substrate characteristics, at the end of the experiment.

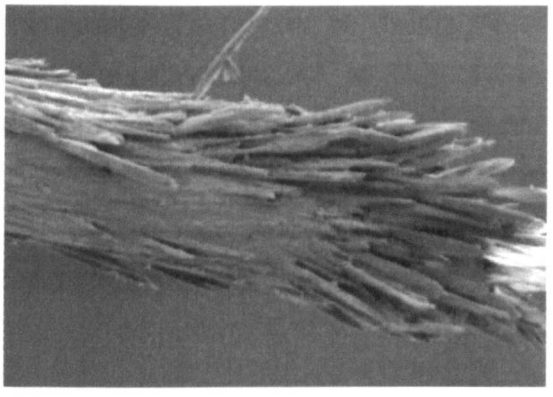

Figure 5.21: SEM image of a hair shaft illustrating damage after 90 days of inoculated composting (mag. x1.0K, 5.0kV).

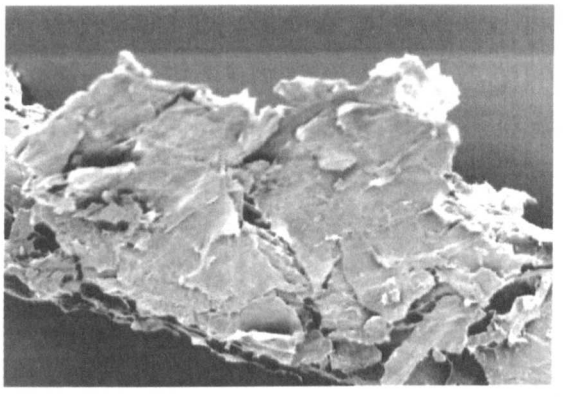

Figure 5.22: SEM image of a hair shaft illustrating damage after 120 days of inoculated composting (mag. x1.0K, 5.0kV).

From the observed microscopic (SEM) images (Figures 5.17 – 5.22) revealing the extent of the structural damage, it was evident that incorporation of the microbial keratinolytic enzyme was beneficial to the enhancement of biodegradation of hair, but it was also noted that enzymatic efficiency of biodegradation may have been impeded or reduced at some stage during the process. A number of factors may have contributed to this:

First, the operational pH was not at optimum pH of activity (pH 9.0) for the isolated microbial enzyme. Operating at pH above 8.5 may have increased the loss of nitrogen through volatilisation of ammonia (Pagans *et al.*, 2006; Stentiford and Pereira, 1985, Vallini *et al.*, 2002), which made it necessary that the composting process be restricted to conditions not higher than pH 8.5.

Secondly, previous studies have indicated that in a composting environment, the inoculated microbial population and indigenous populations would keep evolving continuously, leading to variations in performance during different stages of composting (Xi *et al.*, 2005). Due to the dynamic evolution of the microbial flora within the composting environment the inoculant efficiency may have been affected by competition with the indigenous micro-organisms (Shin *et al.*, 1999). Thirdly, at the initial stages of composting the concentration of the organic

matter is known to be high enough, while microbial population is limited. Thus, inoculation is considered helpful to the composting process at the initial stage. At a later stage, as the microbial population increases, the concentration of the substrate decreases and begins to be a limiting factor (Xi *et al.*, 2005). As previously mentioned (Section 5.4.2.2), biodegradation of the organic material is due to enzymatic action and involves micro-organisms. Ramos *et al.* (1993) observes that biodegradability and the rate of biodegradation is generally dependent on the substrate structure, its composition and the existing micro-organisms. In addition, the rate of biodegradation decreases as the reaction progresses (Ramos *et al.* 1993). This has been suggested to be due to enzyme and substrate-related factors, which includes shearing and thermal inactivation of the enzyme, end-product inhibition, and crystallinity of the substrate (Mansfield *et al.,* 1999; Ramos *et al.* 1993).

5.4.3 Evaluation of compost stability

5.4.3.1 Humification parameters

Humification represents an important process during composting of the organic materials where humic substances are formed while nonhumic substances decompose (Bernal *et al*, 2009; Senesi, 1989). The agronomical value of a compost increases when the organic material reaches a high level of humification. As such, the principal requirement for a compost to be safely and conveniently used as a soil amendment is when the organic matter has attained high degree of stability, or maturity. Consequently, humification parameters have been used to represent compost stability and maturity (Bernal, *et al.*, 2009; Garcia *et al.*, 1991; Jimenez and Garcia, 1989; Senesi and Brunetti, 1996). Although stability and maturity are often used interchangeably, they each refer to specific properties of the material (Bernal *et al.*, 2009). Stability has been described as the degree to which the biodegradable components of the composting feedstock have been biologically decomposed (Gilbert *et al.*, 2001; Lasaridi and Stentiford, 1998). On the other hand, maturity is characterised by germination indexes, that is to say, mature compost should contain no phytotoxic compounds and plant or animal pathogens (Bernal *et al.*, 1998; Iannotti *et al.*, 1993; Zucconi *et al.*, 1981). Respirometric techniques based on the measure of oxygen consumption or carbon dioxide produced, and enzyme activities are among the many methods which have been used for the evaluation of compost stability (Ianotti *et al.*, 1994; Lasaridi and Stentiford, 1998; Tiquia, 2002; Wu and Ma, 2002).

The results of the humification parameters estimated as the Degree of Humification (DH) and Humification Rate (HR) for each of the composting experiments are presented in Tables 5.3 and 5.4, respectively. In each composting experiment the total alkali extracted carbon (TEC) gradually increased with composting time. The total extractable carbon (TEC) for the uninoculated composts ranged from 9.5 – 14.7% (Table 5.3), whereas the TEC of inoculated composts were slightly higher ranging from 10.1 – 15.3% (Table 5.4). The increase in the TEC may be attributed to the increase in carbonaceous materials as composting progressed, this has been observed by other researchers (Mondini *et al.*, 2006; Tomati *et al.*, 1995).

Table 5.3: Humification parameters of Phase 1 uninoculated compost samples at different stages of composting.

Composting period (days)	TEC (%)*	HA (%)*	FA (%)*	HA + FA *	DH (%)	HR (%)
0	9.5 ± 2.0	2.3 ± 0.6	2.7 ± 1.2	5.0 ± 2.0	52.6	8.9
30	11.5 ± 1.5	4.4 ± 0.3	2.7 ± 0.8	7.1 ± 1.2	61.7	13.4
60	12.4 ± 1.3	4.7 ± 1.0	3.4 ± 1.1	8.1 ± 2.2	65.3	15.0
80	13.0 ± 1.0	5.1 ± 1.3	3.6 ± 1.0	8.7 ± 2.5	67.0	16.7
90	13.3 ± 3.0	5.8 ± 0.7	3.4 ± 0.4	9.2 ± 1.2	69.1	18.0
100	13.9 ± 0.5	6.3 ± 1.0	3.8 ± 1.3	10.1 ± 2.4	73.0	21.0
120	14.7 ± 1.1	6.7 ± 1.5	4.2 ± 0.6	10.9 ± 2.2	72.1	26.0

TEC = total extractable carbon; HA = humic acid; FA = fulvic acid; DH = degree of humification; HR =humification ratio. *Mean of four duplicates ± confidence interval.

Table 5.4: Humification parameters of Phase 2 inoculated compost samples at different stages of composting.

Composting period (days)	TEC (%)*	HA (%)*	FA (%)*	HA + FA *	DH (%)	HR (%)
0	10.1 ± 1.8	2.5 ± 1.2	2.9 ± 0.5	5.4 ± 1.8	53.5	9.3
30	11.3 ± 1.1	4.6 ± 0.8	2.7 ± 1.0	7.3 ± 2.0	64.6	15.0
60	12.8 ± 1.3	5.6 ± 1.1	3.3 ± 1.4	8.9 ± 2.6	69.5	18.5
80	14.2 ± 2.2	6.9 ± 0.6	4.1 ± 0.3	11.0 ± 1.0	77.5	22.0
90	14.2 ± 2.6	6.6 ± 0.4	4.5 ± 1.2	11.1 ± 1.7	78.2	24.1
100	15.0 ± 2.0	6.8 ± 2.3	4.7 ± 1.2	11.5 ± 3.6	76.7	26.7
120	15.3± 1.6	7.3 ± 2.0	4.6 ± 1.0	11.9 ± 3.1	77.8	32.2

TEC = total extractable carbon; HA = humic acid; FA = fulvic acid; DH = degree of humification; HR =humification ratio. * Mean of four duplicates ± confidence interval.

Humification parameters based on the determination of carbon content in the fractions (HA and FA) of alkali-extracted humic-like substances and using DH, HR and HI indices has been used to evaluate the stability of organic substrates during composting (Ciavatta *et al.*, 1988; Sequi *et al.*, 1986). As composting progresses the percentage humification is expected to increase relative to the total dry mass or total organic matter (Wu and Ma, 2002). The Degree of Humification (DH %) provides information on the concentration of the humified carbon in the alkali-extracted fraction, whereas Humification Rate (HR %) can provide quantitative information relating to the content of humic substances (HS) with respect to the organic matter (Ciavatta *et al.*, 1990, 1988). The data, in Tables 5.3 and 5.4, shows that both parameters (DH and HR) increased with time at different stages of composting. As shown in Table 5.3, the DH in the uninoculated composts ranged from 52.6 – 73% and HR ranged from 8.9 – 26%. The Degree of Humification (DH) increased by approximately 24% within the first 2 months, suggesting high microbial activity during this period. Thereafter, the DH stabilised for one month between the 60th and 90th day of composting without a pronounced increase, indicating slowing down of substrate transformation. The DH increased to an average peak of 73% at the 100th day, followed by a slight decrease to 72.1% at the final stages of composting.

The humification parameters in inoculated composts also showed increasing trend in both DH and HR values. The DH ranged from 53.5 – 78.2, and HR from 9.3 – 32.2%. The DH increased by 30% in the initial 2 months and reached peak value of 78.2% during the 90th day of composting. In comparison both the DH and HR values of the inoculated composts were significantly ($p < 0.05$) different to uninoculated values which may suggest an accumulation of more humified compounds in the inoculated compost products than in the uninoculated products. Nevertheless, with the end-products from both composting experiments producing values of degree of humification and humification ratios of 73 – 78% and 26 – 32%, respectively; a reasonable stabilisation, comparable with other studies (Mondini *et al.*, 2003; 2006; Tomati *et al.*, 1995) , was attained.

The efficiency of the humification process and stabilisation of the organic matter is further demonstrated by the final values reached by Humification Index (HI) as illustrated in Figure 5.23. The HI values for uninoculated composting experiment ranged from 0.9, at the start of composting, to 0.35 for the final product. On the other hand, the HI values for the inoculated composting experiment ranged from 0.87, at the start of composting, to 0.29 for the final product. The HI values for inoculated composts were, often, slightly lower than the values obtained from the uninoculated compost at different stages during the composting process. Significant difference ($p < 0.05$) between the uninoculated and inoculated composting

experiments was observed between the 60th and 90th day of composting. The low values in inoculated composts further suggests better humification of the organic materials, which may have been due to increased microbial decomposition of the substrate. Nevertheless, in both compost experiments, the final average values reached were 0.35 and 0.29 for uninoculated and inoculated products, respectively, indicating reasonable stability. The threshold value for the HI in well stabilised compost has been suggested as 0.5 (Ciavatta *et al.*, 1990), while Sequi *et al.* (1986) also suggested values < 1.0, for a good degree of stabilisation.

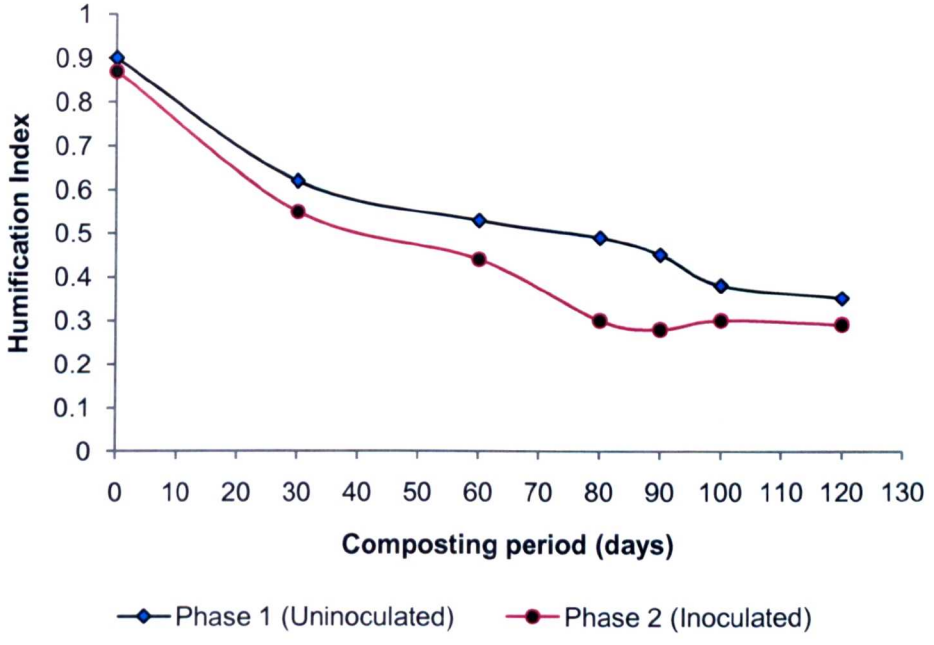

Figure 5.23: A time course profile of the Humification Index (HI) illustrating the extent of the stability of uninoculated and inoculated composting processes.

5.5 Physical and chemical characteristics of the end product

Figure 5.24 represent the physical characteristics of the final compost. The compost was dark, had a tacky feel and an earthy smell, suggesting good quality compost. Microscopic examination revealed that the products from inoculated compost were of smaller and finer particle sizes compared to products from uninoculated compost as illustrated in Figures 5.25 and 5.26. This may suggest that inoculation may have improved the efficiency of breaking down the organic matter.

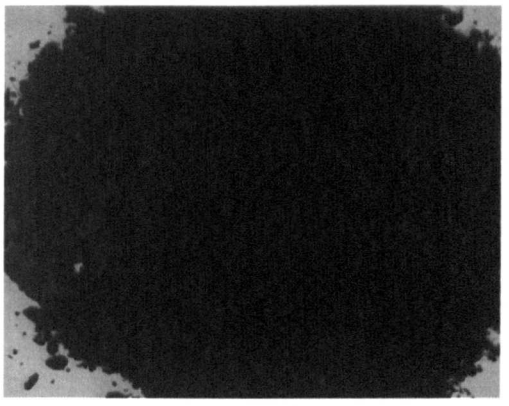

Figure 5.24: Photographic image of the finished compost (end product) following composting of bovine hair for 120 days.

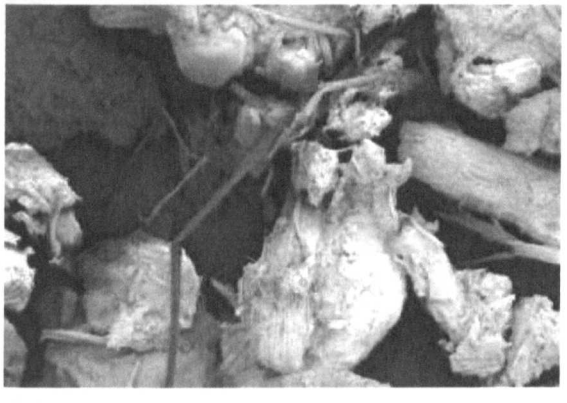

Figure 5.25: A representative (SEM) image of the end product of uninoculated compost illustrating larger particles after composting for 120 days (mag. x40, 5.0kV).

Figure 5.26: A representative (SEM) image of the end product of inoculated compost illustrating finer particles after composting for 120 days (mag. x40, 5.0kV).

The quality parameters of the final compost are summarised in Table 5.5. The pH of the final products was within the range of neutrality (6.7 – 7.7) and an electrical conductivity (EC) of 1.6 – 2.0, which is within the recommended limits. The C/N ratio range of 25 – 29 was higher which may suggest that not all the organic substrates were decomposed due to the crystallinity nature of the substrates (hair and lignocellulosic materials such as leaves and sawdust).

Table 5.5: Summary of the chemical properties of the end products of uninoculated and inoculated composts (values are mean values based on oven dried samples at 105°C).

Parameter	Compost sample	
	Phase 1 (Uninoculated)	Phase 2 (Inoculated)
pH	6.7	7.7
Conductivity (dS m^{-1})	1.6	2.0
Ash (%)	58	54
Carbon (%)	41	37
Total Nitrogen (%)	1.40	1.47
C/N	29	25

5.6 Summary of the Chapter

- In this study three composting experiments were carried out; (1) laboratory-scale static composting, (2) simulated in-vessel rotary drum composting (uninoculated), and (3) simulated in-vessel rotary drum composting (inoculated with crude microbial keratinolytic enzyme). The feedstock mixture comprised of bovine hair, dry leaves and woodchips/sawdust. All composting processes were carried out under a controlled thermophilic temperature range of 40 – 50°C. The process control and monitoring parameters consisted of: (1) temperature control, (2) routine sampling and testing of moisture content and pH, (3) agitation, (4) substrate physical structure changes, and (5) chemical and biochemical parameters, such as changes in carbon and nitrogen ratio, and humification parameters (total extracted carbon, TEC, degree of humification, DH %, humification ratio, HR %, and humification index, HI).

- The laboratory-scale composting results indicated that the feedstock temperature (39 ± 1°C) could not achieve and maintain the expected thermophilic levels. Under static conditions the process was characterised by variability in moisture content, localised accumulation of water within the feedstock, and inadequate aeration leading to poor heat distribution, occasional drying and development of anaerobic conditions. The microscopic examination of the substrate indicated the conditions appeared to favour fungal growth on the surface of the substrate without structural damage.

- The process parameters applied in the rotary drum to simulate an in-vessel composting process indicated that, under the experimental conditions, it was possible to maintain thermophilic range of temperatures (40 – 50°C), moisture content (55 ±

2%) and pH (7.0 – 8.0) favourable for microbial degradation of hair. Fragmentation of the hair structure was achieved after 120 days of composting. The most resistant component was the cuticle.

- The microscopy (SEM) results showed that the use of inoculant enhanced the breakdown of specific components of hair during composting in spite of the fact the level of pH of optimum activity (pH 9.0) was not achieved.

- The results of the monitored changes in carbon to nitrogen (C/N) showed a gradual decrease in both composting experiments. The final values reached for the C/N were 29:1 and 25:1 for uninoculated and inoculated composts, respectively. In comparison the value for the inoculated compost was found to be significant suggesting higher stability than uninoculated compost. Although C/N less than 20 is considered ideal for well stabilised compost this limit may not have been achieved owing to the presence of other recalcitrant substrates such as lignin.

- Humification parameters (DH, HR and HI) have been used to monitor and evaluate the degree of stability of compost. Generally, the humification parameters (DH and HR) showed increasing trends, while HI decreased, as expected in composting processes. A comparison of humification patterns in the two composting experiments (uninoculated and inoculated) showed that during composting, humification parameters (DH % and HR %) of inoculated compost samples were always higher, and HI lower than those of uninoculated products, suggesting an improved decomposition and humification of the organic matter in inoculated process. However, the overall results obtained from the different humification parameters (DH, HR and HI) of the two composting experiments demonstrated that the efficiency of transformation and satisfactory stability was reached for the final product. The final values for the end product were as follows: (DH) degree of humification (73 – 78%), (HR) humification ratio (26 – 32%), and (HI) humification index (0.29 – 0.35).

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

CHAPTER 6

6. CONCLUSIONS

This research was undertaken towards developing a more environmental-friendly sustainable management of tannery hair waste through composting.

Thermal and chemical treatments were carried out, as initial pretreatments, to enhance the digestibility of bovine hair. The results from physiochemical analysis indicated that thermal pretreatment as a single pretreatment strategy was not effective. Chemical pretreatment, involving the use of potassium thioglycolate and sodium hydroxide was found to enhance enzymatic digestibility of bovine hair. However, due to environmental process factors such as pH and microbial growth conditions, these chemicals were considered not suitable to be incorporated during the composting process. Thermal analysis and evidence from the electrophoretic results (SDS-PAGE) showed that, the thermal stability and molecular characteristics of black, brown and white hair were similar. A thermal glass transition temperature was observed at 147±1.8°C, and denaturation temperature of 225±1.9°C. On the other hand, bovine hair proteins were distributed in three major molecular weight band ranges of 48kD – 59kD. The results suggest that melanin does not form part of the molecular structure of bovine hair proteins. However, a comparative solubilisation of black, brown and white with sodium sulfide showed that white was more vulnerable to sulfide as compared to melanised hair samples.

Enzymatic and microbial degradation of bovine hair were studied. Based on the results of the concentration of hair protein digested and structural changes analysis by scanning electron microscope (SEM), the degradation of hair was enhanced in a combined protease treatment consisting of proteinase K and trypsin enzymes. The presence of micro-organisms with the potential to degrade keratin was demonstrated. The predominant micro-organism was identified to belong to the bacterial species *Bacillus*. The microbial extracellular enzyme was partially purified. The microbial enzyme observed to exhibit both proteolytic and keratinolytic activity at a broad pH range of 7.5 – 10, with an optimum activity at pH 9. Optimum activity was found to be at 50°C. The modification of the hair structure due to enzymatic hydrolysis and microbial action was found to be selective. The selective degradation of the hair structure was found to be initiated from the cell membrane complex (CMC), proceeding through the δ-layer and the endocuticle region. The medulla, cytoplasmic

materials and fibrillar components of the cortex were degraded in that order. The highly keratinised cortical fibres and the cuticle were more resistant to enzymatic and microbial degradation. Microbial degradation involved both enzymatic action and mechanical splitting of the hair structure. Synergistic action of the different microbial enzymes was considered to be an important mechanism by which micro-organisms degraded the complex hair structure.

Composting of bovine hair was carried out in a tannery rotary drum, simulating an in-vessel composting process for 120 days. The biodegradation of bovine hair was successful under the controlled composting environment of moisture content (55 ± 2%), pH (7 – 8), and at a thermophilic range of temperatures (40 – 50°C). The thermophilic temperatures and the moisture content were found favourable in sustaining bacterial growth and metabolic activities. Microscopy (SEM) analysis of the substrate showed that, fragmentation of hair was achieved by the final day of composting. Further microscopy (SEM) results of the structural changes of the substrate showed that, the introduction of the isolated microbial enzyme as inoculant during the controlled composting process, improved the biodegradation of hair. The structural components of hair, such as medulla and the cortex were completely degraded. The cuticle was the most resistant to undergo degradation. However, it can be concluded that, this group of micro-organisms and their enzymes offers the potential for improved bioconversion of tannery hair waste into useful products, such as compost, at industrial scale.

The stability of the compost, based on the values of the ratio of carbon to nitrogen (C/N), degree of humification (DH), humification ratio (HR), and humification index (HI), showed that the final product reached high stability at the end of the composting process. These results demonstrate that under the appropriate conditions bovine hair waste from the tannery can be composted, and that composting may be considered as a more sustainable and environmentally-friendly technology for the treatment of solid hair waste.

The results further demonstrate that, the composting technology can fit within existing tannery facilities, thus providing the industry with an overall waste management plan that is economically viable, environmentally-sound, as well as contributing to sustainable development.

7. FUTURE WORK

The tanning industry has been characterised as a highly polluting industry. Worldwide, the industry still faces major challenges in dealing with substantial amount of solid waste. Although this research has bridged the gap by demonstrating the availability of composting as an environmentally-friendly alternative technology for the recycling solid hair waste, future challenges that could be undertaken for further investigations include:

- Manipulation of the micro-organisms responsible for the degradation of highly resistant protein substrates so as to improve their potential and stability during the composting process.

- Designing appropriate composting facilities to allow process controls that will optimise environmental conditions for the desired micro-organisms. Maintaining optimum environmental conditions will maximise the degradation of the substrate, ensuring consistency and good quality of the end-product.

- Although this research studied the ability of bovine hair to be efficiently composted, future studies should consider the possibility of composting mixed tannery wastes. This will be a more attractive and economically-viable investment for the tanners.

- Agronomical studies on the potential of the end-product should be undertaken to evaluate and provide evidence of the potential value of the final compost as fertiliser.

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APPENDICES

Determination of Total Kjeldahl Nitrogen

Protocol

Bovine hair sample (1 g) was weighed on laboratory analytical balance and transferred into a dry 800 ml Kjeldahl flask containing 9 g of Kjeldahl catalyst mixture (100 g copper sulfate and 1000 g sodium sulfate) (Fisher Scientific, UK). Concentrated sulfuric acid, 20 ml, was added to the flask. Samples were placed on the digestion rack in fume cupboard and digested until the mixture turned clear and then one hour longer. The blank control contained the catalyst mixture and acid. After the samples were cooled 250 ml of deionised water and some anti-bumping granules was added plus three drops of silicon anti-foaming agent (BDH, UK). The samples were placed on the distillation apparatus.

Saturated boric acid, 100 ml, was placed into a 500 ml conical flask and a few drops of screened methyl red indicator added to it. The flask was then placed at the receiving end of the condenser. A 30% sodium hydroxide (85 ml) was added to the Kjeldahl flask containing samples and the condenser water turned on. The solution was boiled vigorously until the volume in the receiving flask containing boric acid reached 300 ml then the condenser water and heat turned off. The distillate was titrated with 0.2 M hydrochloric acid to end point, noted by the purple colour. The percentage nitrogen was calculated according to the Equation below. The determination was carried out in duplicate.

$$\% \text{ N} = \frac{(b - a) \times N \text{ of acid} \times 0.014}{\text{Weight of sample (g)}} \times 100$$

Where a = volume of acid used in blank titration (ml)

b = volume of acid used in sample titration (ml)

N = exact concentration of acid (Moles)

Ash content

Protocol

Duplicate samples (1 ± 0.2 g) of oven dried (105°C) bovine hair, were placed in silica dishes which had been preheated in a muffle furnace at 550°C and cooled in a desiccator to a constant weight. Concentrated sulfuric acid (5 ml) was added and the sample digested in fume cupboard before being transferred to a muffle furnace and allowed to ignite at 550°C for 16 hours. The samples were cooled in a desiccator to a constant weight. Ash content in the sample was calculated by weight difference using the Equation below.

$$\text{Ash (\%)} = \frac{W_2 - W_1 \text{ X } 100}{W\,(\text{g})}$$

Weight of empty silica dish = W_1

Final weight of sample plus silica dish after cooling to constant weight (g) = W_2

Weight of oven dried sample (g) = W

Determination of extractable lipids

Protocol

Bovine hair sample (6 ±1 g dry weights) was weighed and placed into a 30 x 100 mm cellulose Soxhlet extraction thimble and plugged with cotton wool (to prevent the evaporation of extracting solvent). A 250 ml flat bottomed flask (B34 neck) containing some anti-bumping granules, previously dried and cooled in a desiccator was weighed on laboratory analytical balance prior to analysis. Dichloromethane, 200 ml, was added into the flask and the thimble containing the sample inserted into the Soxhlet extractor. The condenser water was turned on and the extraction carried out for 4 hours. After the extraction, the dichloromethane was recovered by distillation and the flask placed in an oven at 102°C for one hour to evaporate the excess dichloromethane. The flask was cooled in the desiccator and then reweighed. The percentage lipid extracted was determined by weight difference, calculated from the Equation below.

$$\text{Extractable lipids (\%)} = \frac{W_2 - W_1}{W(\text{g})} \times 100$$

Weight of empty flask (g) = W_1

Weight of flask plus extracted lipids after oven drying at 102°C = W_2

Weight of sample (g) = W

Protein determination

Reagents

1% (w/v) $CuSO_4.5H_2O$ (Fisher Scientific, UK), in deionised water
2% (w/v) Na Tartrate (Fisher Scientific, UK), in deionised water
2% (w/v) Na_2CO_3 in 0.1 M NaOH (Fisher Scientific, UK).
Folin-Ciocalteu Reagent (Sigma Aldrich, UK): used at 1 N concentration.
Bovine serum albumin (BSA) (Sigma Aldrich, UK)
Sample solution of unknown protein concentration

Preparation of alkaline copper reagent (prepared fresh, total volume 100 ml, 1 hour before use).

- Mix 1 ml of 1% (w/v) $CuSO_4.5H_2O$ and 1 ml of 2% (w/v) Na Tartrate
- Add 98 ml of 2% (w/v) Na_2CO_3 in 0.1 M NaOH

Preparation of protein standards and sample of unknown protein concentration were carried out as follows:

- A series of glass tubes containing 10 – 300 µg/ml protein standards (BSA) were prepared using deionised water in final volumes of 1.2 ml. A blank contained 1.2 ml H_2O. All the protein standards and blank solutions were prepared in duplicate.
- Appropriate dilution of the protein solution of unknown concentration was carried using deionised water. The diluted protein solution of unknown concentration (1.2 ml) was placed in separate duplicate glass tubes.

Assay protocol

- Alkaline copper reagent (6 ml) was added into each tube and mixed using a rotary mixer.
- After 10 minutes at ambient conditions, Folin-Ciocalteu reagent (0.3 ml) was added and immediately mixed using a rotary mixer.
- The test solutions were incubated at ambient temperatures for 30 minutes.
- After 30 minutes the absorbance of the protein standards and protein solution of unknown concentration was read at 500 nm against the blank on UV 2501PC spectrophotometer (Shimadzu, Japan). The concentration of protein in the unknown test solution was estimated from the protein standards curve.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel composition and preparation

Resolving gel (15%)

Reagent	Volume (ml)
30% acrylamide	5.0
1 M Tris-HCl buffer, pH 8.8	7.5
10% SDS	0.2
Deionised water	4.0
1.5% ammonium persulfate	0.6
TEMED	0.112

Stacking gel (4%)

Reagent	Volume (ml)
30% acrylamide	2.66
1 M Tris-HCl buffer, pH 6.8	2.6
10% SDS	0.2
Deionised water	5.0
1.5% ammonium persulfate	0.6
TEMED	0.04

Electrophoresis buffer, pH 8.6

25 m M Tris

1.44% glycine

10% SDS

10% SDS

Fixation of the gels

12.5% (w/v) trichloroacetic acid (TCA): 30 minutes

Gel staining

0.2% (w/v) Coomassie Brilliant Blue R-250

40% (v/v) methanol

17% (v/v) acetic acid

Destain solution

10% (v/v) methanol
7% (v/v) acetic acid

SDS-PAGE: Optimisation studies

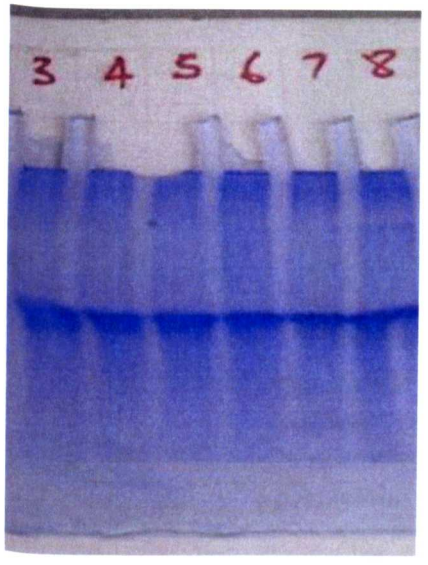

Figure E1: SDS-PAGE of non *S-carbamoylmethylated* bovine hair keratins (SCams). Lane (3 & 4): white hair, (5 & 6): black hair, and (7 & 8): brown hair

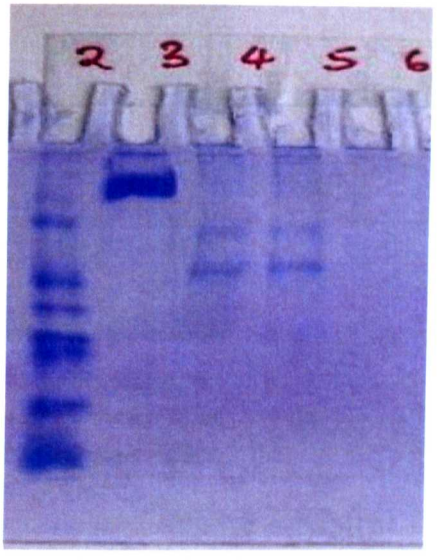

Figure E2: SDS-PAGE of *S-carbamoylmethylated* bovine hair keratins (SCams) in a 10% (v/v) polyacrylamide gel. Lane (2): standard, (3): high molecular weight standard, (4): white hair, (5): black hair, and (6): brown hair

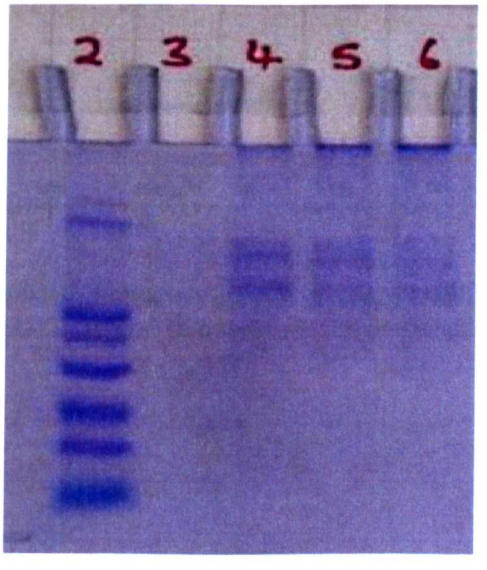

Figure E3: SDS-PAGE of *S-carbamoylmethylated* bovine hair keratins (SCams) in a 15% (v/v) polyacrylamide gel. Lane (2): standard, (3): buffer, (4): white hair, (5): black hair, and (6): brown

Characteristics of the hair samples at glass transition and denaturation temperatures.

Figure F1: Physical conditions of hair samples as observed during thermal measurement on the DSC at temperature range 20 – 230°C. Samples were carbonised by the end of he DSC run at 230°C.

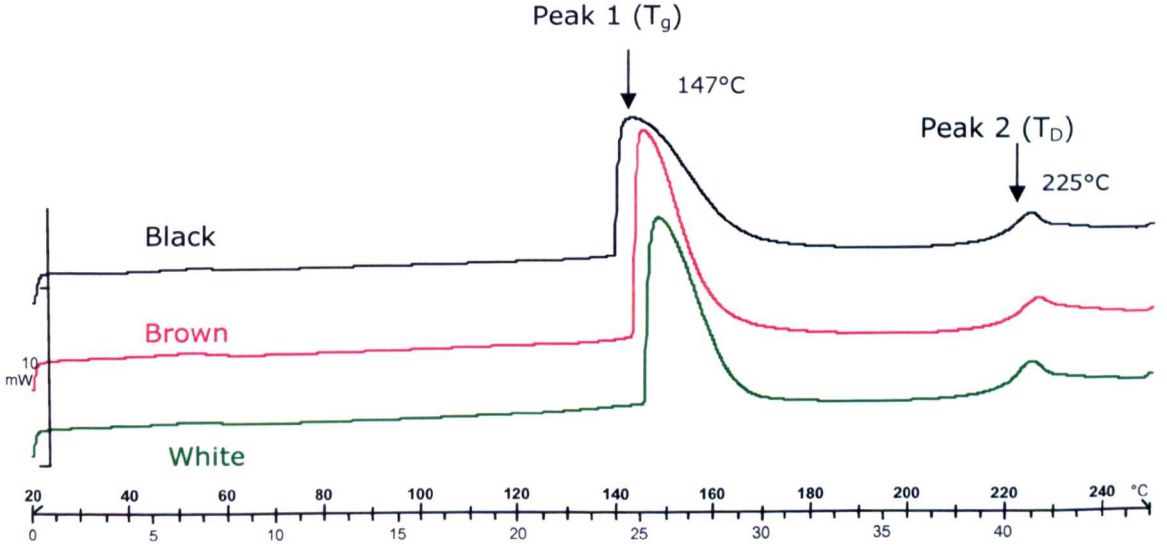

Figure F2: DSC thermal temperature transition curves of intact black, brown and white bovine hair (control). measured from 20 – 250°C: (mean temperature shown on the curve).

Composition and preparation of the *Bacillus* selective agar media

Polymyxin Pyruvate Egg Yolk Mannitol agar (CM0617, Oxoid, UK).

Chemical Formula	gram/litre
Peptone	1.0
Mannitol	10.0
Sodium chloride	2.0
Magnesium sulphate	0.1
Disodium hydrogen phosphate	2.5
Potassium dihydrogen phosphate	0.25
Bromothymol blue	0.12
Sodium pyruvate	10.0
Agar	15.0
pH of the media 7.2 ± 0.2	

Polymyxin B Supplement (SR0099, Oxoid, UK).

Vial contents	Per vial	Per litre
Polymyxin B	50,000 IU	100,000 IU

One vial of polymixin B was added in 500 ml of sterilised agar solution, before pouring into sterilised Petri dishes.

Composting vessels

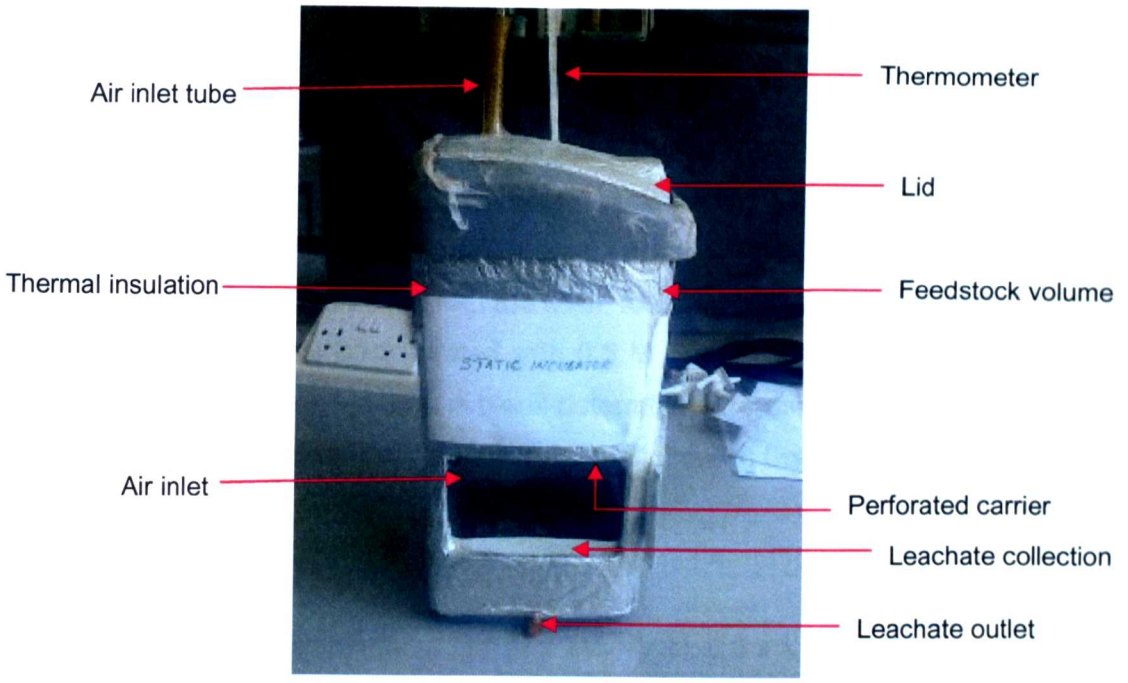

Figure H1: Photographic image of the laboratory-scale composting bin.

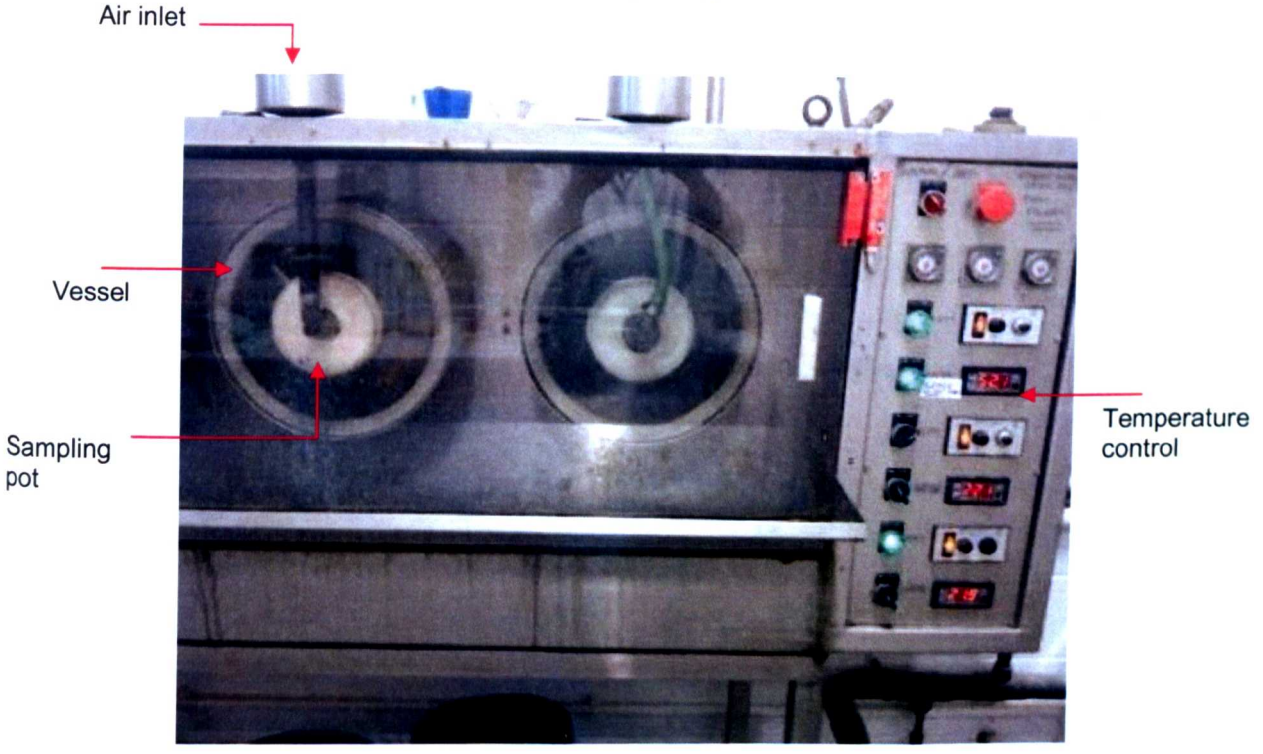

Figure H2: Photographic image of the rotary drum used during the in-vessel composting.

Walkley-Black Method for the Determination of organic carbon (Allison, 1965)

Procedure

Potassium dichromate (1 M $K_2Cr_2O_7$) (10 ml) was added to the alkali (0.1 M NaOH) and sodium pyrophosphate (0.1 M $Na_4P_2O_7$) extracted sample (25 ml) in a 500 ml Erlenmeyer flask and mixed by swirling gently. Concentrated sulfuric acid (20 ml) was added and mixed by swirling vigorously for 1 minute. The flask was allowed to cool by standing for 30 minutes. Deionised water (200 ml) was added into the flask, followed by 3 to 4 drops Ferroin indicator. The excess dichromate was back titrated with 0.5 M Ferrous sulfate ($FeSO_4.7H_2O$) to end point, marked by a dark green colour. A blank determination was carried out using deionised water. The percentage carbon was calculated by the following Equation.

$$\text{Organic carbon (\%)} = \frac{M \times (V_2 - V_1) \times 0.3 \times 1.33}{W}$$

Where M = molarity of the $FeSO_4.7H_2O$

V_1 = volume (ml) of $FeSO_4.7H_2O$ required to titrate the blank.

V_2 = volume (ml) of $FeSO_4.7H_2O$ required in actual titration of the sample.

W = weight of the soil sample (g).

1.33 = correction factor.